

PROGNOSTIC BIOMARKERS FOR PROSTATE CANCER

GISÈLE LEYTEN

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The work presented in this thesis was performed at the Department of Urology, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands.
A part of this research was performed with grants from PCMM (03-O-203), EFRO and Ultrasense MR (EFRO: 2010-01317).

Publication of this thesis was financially supported by: Astellas Pharma B.V., Bayer B.V., Chipsoft B.V., DDL Diagnostic Laboratory, GlaxoSmithKline B.V., Ipsen Farmaceutica B.V., Janssen-Cilag B.V., NovioGendix B.V., Olympus Nederland B.V., Pfizer B.V., Pohl Boskamp, Sanofi Aventis Nederland B.V., Zambon Nederland B.V.

ISBN/EAN 978-90-9028589-4

Cover design & Lay-out: Esther Beekman (www.estherontwerpt.nl)
Printed by: Ipskamp drukkers BV, Enschede, the Netherlands

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Prognostic biomarkers for prostate cancer

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. Th.L.M. Engelen,
volgens besluit van het college van decanen
in het openbaar te verdedigen op maandag 8 december 2014
om 16.30 uur precies

door

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geboren op 19 september 1983
te Dronten

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Manuscriptcommissie:

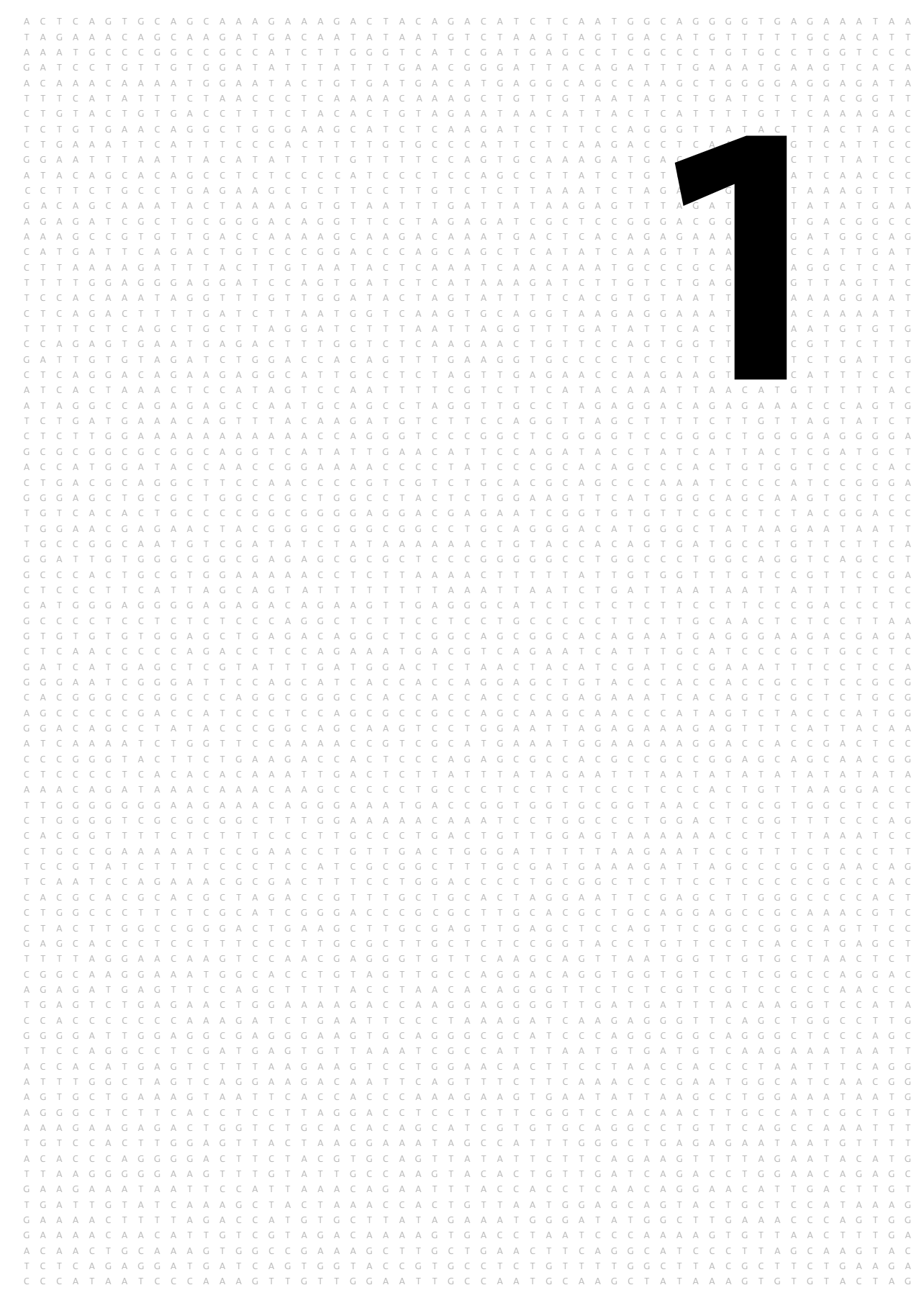
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General introduction

The prostate is an exocrine gland, located beneath the urinary bladder in men. It is walnut-sized and produces prostatic fluid which plays a role in the function of seminal fluid. The volume of the ageing prostate will gradually increase caused by benign prostate hypertrophy (BPH). But also a malignancy of the prostate can develop, this is called prostate cancer.

Prostate cancer is the second most commonly diagnosed cancer in men, accounting for 14% of all cancers in men¹. The gold standard for the diagnosis of prostate cancer is based on the histopathological evaluation of prostate biopsies, an invasive and unpleasant procedure with significant morbidity (i.e. haematuria and urosepsis). Localized prostate cancer often does not present with symptoms, therefore, the selection of men qualifying for prostate biopsies relies on serum PSA (prostate specific antigen) testing and digital rectal examination (DRE).

However, DRE findings are only moderately reproducible^{2,3}, and tends to diagnose prostate cancer in a more advanced stage and therefore less likely to be curable⁴. Furthermore, PSA has a low specificity of 25-40% in the so-called 'grey area' of PSA levels 4.0-10.0 ng/mL, resulting in a high negative biopsy rate^{5,6}. Moreover, widespread PSA testing has led to the diagnosis of clinically insignificant prostate tumours, resulting in potential overtreatment. Therefore, the need for more specific and prognostic biomarkers persists.

OUTLINE OF THE THESIS

Chapter 2 gives a short history of prostate cancer biomarkers, an overview of the established prostate cancer biomarkers PSA and PCA3; and promising novel prostate biomarkers (tissue markers, blood markers and urine markers): TMPSS2-ERG, Ki-67/MIB1-Labeling Index, PTEN, E-cadherin, EZH2, neuroendocrine phenotype, microRNAs and circulating tumour cells (CTCs).

Multiparametric 3 tesla MRI is being used increasingly in the diagnostic process of prostate cancer. This MRI examination consists of anatomic (T2W) images, dynamic contrast-enhanced (DCE) MRI, diffusion weighted imaging (DWI) and proton MR spectroscopic imaging (MRSI). It has a high accuracy to detect prostate cancer, if read by an experienced radiologist. However, due to additional expenses and limited availability compared to the conventional TRUS-guided biopsy, the routine application of MRI-guided biopsy is not yet feasible. An additional, cheaper and more practical test to select patients who require multiparametric MRI, and potentially subsequent MR-guided biopsy, would

be valuable. In **chapter 3** we evaluated the clinical value of the Progens PCA3 test: its predictive value for biopsy outcome, Gleason score and MRI outcome. This was evaluated in a retrospective study of 591 men who underwent a Progens PCA3 test at the Radboud University Nijmegen Medical Centre.

Considering the heterogeneous character of the disease, the use of a biomarker panel can further improve the diagnostic accuracy. In **chapter 4**, we present the results of a large prospective multicenter study that evaluated the diagnostic and prognostic value of Progens PCA3 and TMPRSS2-ERG gene fusions for prostate cancer, as individual markers and as a biomarker panel.

Progens PCA3 aids in the diagnosis of prostate cancer. However, its prognostic value seems limited. Therefore, biomarkers that can be used to identify patients with clinically significant disease are urgently needed. To address this unmet need, the stepwise development of a four gene based urinary test is described in **chapter 5**. This test was evaluated in a prospective multicenter study on urine specimens of 358 men undergoing prostate biopsies.

Patients with advanced prostate cancer who undergo chemical or surgical castration will ultimately experience a relapse, this state is called castrate-resistant prostate cancer (CRPC). Treatment options for CRPC patients are limited. The main issue in the management of CRPC and development of new therapies is the lack of surrogate endpoints for treatment response and survival. Circulation tumour cells (CTCs) in the peripheral blood has been evaluated as a potential prognostic marker and predictor of overall survival. Little is known about prostate cancer specific biomarkers in CRPC patients as a surrogate test for CTCs. In **chapter 6**, we demonstrate the results of an exploratory study for the feasibility of a highly sensitive modified nucleic acid amplification assay to assess KLK3, PCA3 and TMPRSS2-ERG mRNA in the peripheral blood mononuclear cell fraction from CRPC patients.

Future perspectives are discussed in **chapter 7**.

A summary is presented in **chapter 8**.

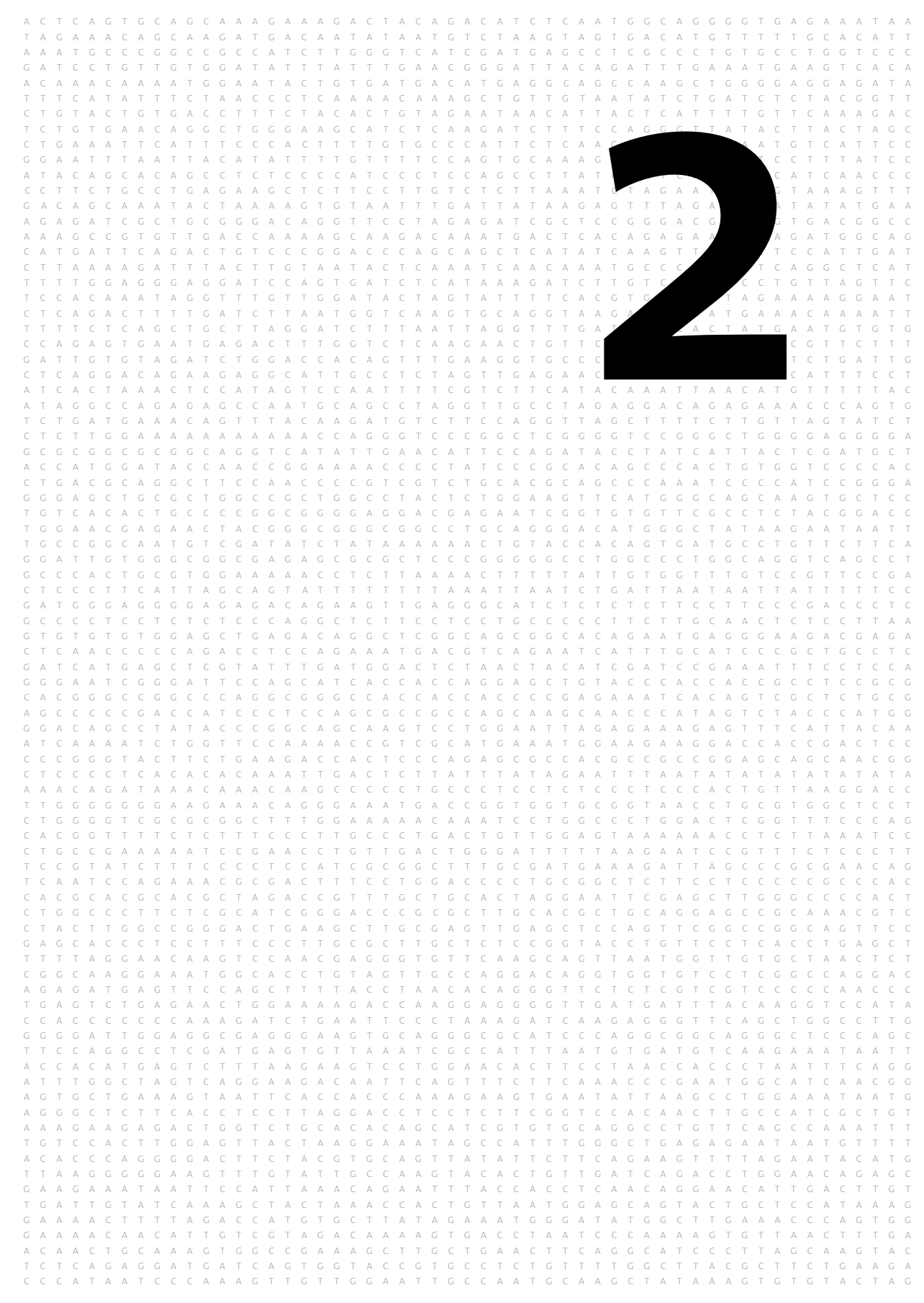
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Biomarkers for prostate cancer

Adaptation of:

Management of prostate cancer, Springer, 2012.

Editors: Michel Bolla and Hendrik van Poppel.

Chapter 2 - Biomarkers for Prostate Cancer.

Gisele H.J.M. Leyten, Peter F.A. Mulders, Jack A. Schalken

Traditionally, clinical diagnosis and management of the individual patient are based on clinical cohort based studies. The heterogeneity within 'risk cohorts' can still be considerable which impairs decision making for an individual patient. Therefore, we urgently need improved methods to accurately predict the biological behaviour, and therapy response for well stratified/homogeneous groups of patients. In the last decade revolutionary advancements in molecular profiling technologies have been made resulting in new diagnostic algorithms. It is noteworthy that it is just 60 years ago that the double-helix model for the structure of DNA was first described. Molecular biology developed quickly and with nucleic acid amplification technologies whole genome gene and expression profiling became feasible. The field expanded beyond the traditional/core genes that follow Francis crick's dogma (gene \leftrightarrow RNA \rightarrow protein) by the discovery of non coding RNAs, including microRNAs. This enables us to identify the individual in a different way from the way we did before. These advances have marked the beginning of a new era for modern medicine: individualized medicine. This is an approach that strives for a 'customized' healthcare. Patient-specific strategies instead of the standard 'one-size-fits-all' approach.

Biomarkers are important tools in individualized medicine. A biomarker can be defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention¹. This includes physiological measurements and clinical imaging, but also specific cells, molecules, genes, gene products, enzymes or hormones.

Biomarkers in cancer (can) have several valuable applications:

- improve diagnosis
- improve staging
- indicate disease prognosis (e.g. indolent vs. clinical significant prostate cancer)
- monitor response to treatment
- select patients for different treatment options
- surrogate endpoint in trials
- therapeutic target

In prostate cancer, prostatic acid phosphatase (PAP) is considered the first known biomarker. This enzyme was discovered to be increased in men with metastasized prostate cancer in 1938². The use of PAP was not useful for diagnosis, and was only used to monitor prostate cancer patients after diagnosis. In the 1980s, prostate specific antigen (PSA) was introduced into clinical practice. This is to date the only widely used

biomarker in prostate cancer. The introduction of PSA has resulted in earlier detection of the disease, but also has important limitations. Its use in screening and prognosis remains controversial. Novel biomarkers are needed to differentiate indolent from aggressive disease to minimize overtreatment of clinically insignificant prostate cancer.

The ideal characteristics of a biomarker for prostate cancer are:

- Only produced by tumour tissue
- Non-invasive test, easy to manage
- As inexpensive as possible
- Ability to detect prostate cancer at an early stage
- Differentiate between indolent and clinically significant tumours
- High sensitivity and specificity

Given the heterogeneous character of prostate cancer, it is most likely that in the future a panel of (novel) biomarkers will be used to optimize predictive value. Prostate cancer biomarkers can be detected in different diagnostic substrates, each aiding different clinical decisions (Table 1).

Novel biomarkers can be identified through genetic epidemiological studies (evaluating inherited genetic predispositions in large cohorts, Genome Wide Association Studies, GWAS) or molecular profiling studies, evaluating the molecular profile of the tumour. The GWAS studies have revealed at least genetic loci that are associated with an increased chance to develop prostate cancer. The observed relative risks are insufficient to individualize diagnosis³, yet may be of use for pre-selection. This chapter will focus on established biomarkers and promising novel biomarkers identified by molecular profiling studies, arranged by tissue markers, blood markers and urine markers.

Table 1 Different diagnostic substrates for prostate cancer biomarkers

Diagnostic substrates	Invasive	Clinical decision
Urine	-	Biopsy
Blood	-	Biopsy
Biopsy specimen	+	Treatment
Prostatectomy specimen (Gleason score + pTNM)	++	Adjuvant treatment

TISSUE MARKERS

Once tissue is available, important decisions have already been made, either a biopsy has been taken or the gland was surgically removed. Thus, the main clinical need is to accurately predict the biological behavior of the malignant process. In case the pathologist is not sure about the diagnosis of invasive prostate cancer, immunohistochemistry using antibodies against the basal cell specific high molecular weight keratins (34 β E12) and AMACR has proven to be helpful⁴. It is striking that this is the only molecular pathological application that has been widely accepted and used in prostate cancer. Numerous studies report on the potential of biomarkers detected by immunohistochemistry, yet none is routinely used for a better assessment of prognosis. Whereas, for other malignancies biomarkers that predict progression of the disease in patients that were treated with curative intent are routinely used (e.g. breast- and colon cancer), so far there has not been a great interest in adjuvant treatment of patients with high risk localized prostate cancer. The most significant study in this respect was the EPC initiative, in which a stratification on standard clinical and pathological risk factors was used. Now better treatment modalities become available, adjuvant strategies are likely to be considered again and biomarkers indicative for biological behaviour, determined in tissue will be needed. In this part we will focus on high potential biomarkers for which standardized methods are or can be developed.

Gene fusions: TMPRSS2-ERG

The classic example of a gene fusion implicated in cancer development is the BCR:ABL fusion in patients with chronic myelogenous leukemia. This fusion results from a reciprocal translocation T(9;22), first recognized as the Philadelphia chromosome. This discovery has been revolutionary as it has led to the development of imatinib⁵. This is an inhibitor of the BCR:ABL gene fusion product which transformed the previously fatal leukemia into a manageable chronic disease for many patients.

In prostate cancer, recurrent gene rearrangements were discovered in 2005: a fusion of the 5' untranslated region of TMPRSS2 (androgen-regulated trans-membrane protease, serine 2) to Ets family genes (oncogenic transcription factors)⁶. Oncogene ERG (v-ets erythroblastosis virus E26 oncogene homolog (avian)) is the most commonly involved Ets family member in gene fusion. TMPRSS2-ERG has been detected in approximately 50% of Caucasian prostate cancer patients. This gene fusion is less frequently seen in men from other ethnic background. A recent study reported fusion positive prostate cancers in 31% of African American men and only in 16% of Japanese men⁷. Rearrangements with other Ets transcription factors have been identified in approximately 5-10% of PSA

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screened prostate cancers: ETV1 (ETS variant 1 gene), ETV4 and ETV5⁸⁻¹⁰. In addition to TMPRSS2, other fusion partners involved in ETS fusions have been identified. Their possible clinical relevance is not clear.

2

As a result of gene fusion with TMPRSS2, the expression of ERG becomes androgen regulated and thus overexpressed. ERG expression can be detected in prostate cancer patients by immunohistochemistry with a high specificity of >95% and is not seen in benign prostate epithelium^{11,12}. This suggests ERG immunostaining could be a solid diagnostic biomarker, albeit in approximately half of the prostate cancer patients. The clinical relevance of Ets gene fusions is currently under investigation. Results on a potential prognostic value are conflicting. A worse prognosis of fusion positive cancers has been reported by several studies¹³⁻¹⁵. Other studies could not validate these results^{16,17} or found a favourable prognostic association^{18,19}. A recent large study showed that ERG status had no influence on the risk of PSA recurrence after radical prostatectomy¹². In addition, they report a strong association between ERG positivity and high androgen receptor expression levels. This suggests that ERG status might have predictive value for response to anti-androgen therapy. However, this requires further investigation, before implementation into clinical practice can be realized.

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Ki-67/MIB1-labelling index

Expression of the Ki-67 protein is strictly associated with cell proliferation. Ki-67 has therefore been extensively studied for its potential use as a proliferation marker in different types of cancer, including prostate cancer. Its name is derived from the city of origin (Kiel) and the number of the original clone in the 96-well plate²⁰. Ki-67 can be determined by immunohistochemistry using the monoclonal antibody MIB-1.²¹ The proportion of tumour cells staining positive for Ki-67 is known as the Ki-67 labeling index. This proved to be an independent and significant prognostic biomarker for prostate cancer-specific survival^{22,23}. Furthermore, the Ki-67 labeling index has repeatedly shown to be a predictive marker for disease recurrence and progression after radical prostatectomy and radiotherapy²⁴⁻²⁶. Although its usefulness has been well established, the Ki-67 labeling index is currently not used in daily practice.

PTEN

PTEN (Phosphatase and TENSin homologue) is a tumour-suppressor gene, located on chromosome 10q23²⁷. This gene plays a key role in carcinogenesis. PTEN antagonizes the PI-3K/Akt pathway and thereby modulating cell growth/survival and cell migration/adhesion²⁸. In prostate cancer, PTEN loss has been associated with proliferation and

survival of cancer cells, resistance to castration²⁹, chemotherapy^{30,31} and radiotherapy³², bone metastasis³³ and recurrence after radical prostatectomy³⁴. Thus, PTEN is assumed to be a potent prognostic marker and a clear target for novel (gene) therapies. However, this requires further research.

E-cadherin

Cadherins are a family of epithelial cell-cell adhesion molecules that play a key role in preserving epithelial integrity³⁵. Their function is dependent on calcium, hence their name ('calcium-dependent adhesion'). E-cadherin is the most extensively studied member of the cadherin family. During cancer progression to an invasive state, intercellular adhesions between tumour cells are disrupted. Thus, aggressive tumour cells were hypothesized to have loss of E-cadherin. And indeed, decreased E-cadherin expression has repeatedly been shown to correlate with a loss of tumour differentiation and a poor prognosis³⁶⁻³⁸. This correlation has been shown for several tumour types, including prostate cancer. However, large prospective studies will have to define its potential clinical relevance in prostate cancer, as a prognostic biomarker or as a molecular target for therapy.

EZH2

The EZH2 gene (Enhancer of zeste homolog 2), encoding a Polycomb-group (PcG) protein, is responsible for maintaining the silent state of genes. EZH2 mediates trimethylation of histone H3 lysine 27 (H3K27), leading to repression of transcription and thereby silencing of gene expression^{39,40}. EZH2 is upregulated in various aggressive tumours, including prostate cancer⁴¹⁻⁴³. Furthermore, it mediates transcriptional silencing of the tumour suppressor gene E-cadherin⁴⁴. This demonstrates an inverse correlation between dysregulation of EZH2 and repression of E-cadherin during cancer progression. In conclusion, EZH2 upregulation might play a key role in oncogenesis and progression of cancer. This makes it a promising biomarker of disease progression and a viable target for therapeutic interventions in aggressive cancers.

The neuroendocrine phenotype

The expression of a neuroendocrine phenotype in prostate cancer has been reported almost 25 years ago⁴⁵. There is in good agreement that the relative fraction of cells with a NE phenotype increases in advanced prostate cancer, yet the use to predict biological behavior in localized prostate cancer remains controversial. Only in case of a 'pure' NE phenotype, in small cell prostate cancer, a rare entity (<1 % of all prostate cancer), the biology of the disease is markedly different from adenocarcinoma of the prostate, and

therefore, treatment of this type of prostate cancer is different. In summary, we can conclude that a robust set of candidate prognostic biomarkers is available that can be measured by immunohistochemistry. Stratification of patients based on these markers is well within reach provided the methods and scoring systems are standardized.

BLOOD MARKERS

Kallikreins

Total PSA

In 1986, PSA was approved by the Food and Drug Administration as a marker to monitor treatment in patients with prostate cancer, and in 1994 as a diagnostic marker. It is currently the only widely used marker for prostate cancer.

PSA, also known as kallikrein 3 or hK3, is a serine protease that is a member of the family of glandular kallikrein-related peptidases. The genes for the glandular kallikreins are clustered at chromosome 19q133-4 and transcription of PSA is regulated by androgens⁴⁶. The function of PSA is to liquefy seminal fluid through its action on the gel-forming proteins semenogelin and fibronectin⁴⁷.

PSA is not a cancer-specific marker, as it is produced by both benign and malign prostate epithelial cells. Normally, PSA blood levels are low. A healthy prostate is surrounded by a continuous layer of basal cells and a basement membrane which prevent the high concentrations of PSA in the prostate to leak into blood. High PSA blood levels can be caused by an elevated synthesis or an increased release of PSA into blood. An elevated PSA synthesis can be a result of benign prostatic hypertrophy (BPH) and prostate manipulation^{48,49}. PSA expression, ergo PSA synthesis, is slightly decreased in the development and progression of prostate cancer⁵⁰. Therefore, as is seen in prostatitis, the increased PSA blood levels in prostate cancer are assumed to be a result of an increased release of PSA into blood through the disrupted architecture of the prostate.

Despite extensive research, difficulty persists in defining the optimal cutoff value for PSA. Traditionally, it was set at 4.0 ng/ml. Using this PSA cutoff provides a sensitive test, with a positive predictive value of 37% and a negative predictive value of 91%⁵¹. In other words, 75% of men with PSA 4.0-10.0 ng/ml who undergo biopsy do not have cancer⁵². In addition, several studies showed a substantial probability of prostate cancer within the PSA interval 0-4.0 ng/ml⁵³⁻⁵⁵. The Prostate Cancer Prevention Trial (PCPT), for

example, reported that 27% of men with normal DRE and a serum total PSA between 3.1-4.0 ng/ml have prostate cancer⁵⁵. On the other hand, it has never been demonstrated that lowering the PSA cutoff affects the long-term survival in men with prostate cancer. Furthermore, this will most likely lead to a higher number of unnecessary biopsies and an increased detection of clinical insignificant prostate cancer. Other factors of influence on PSA blood level is ethnic background and the use of medication. Men from African descent have higher PSA levels than Caucasian men, even after adjusting for prostate volume^{56,57}. And men using 5 α -reductase inhibitors for treatment of BPH (such as dutasteride and finasteride) will have lower PSA levels by an average of 50% after 6 months of treatment^{58,59}.

Several studies report that PSA measured before age 50 might be indicative for the risk of developing prostate cancer years, or even decades later^{60,61}. It is also suggested that total PSA level at age 44-50 might also predict the likelihood of developing advanced prostate cancer, defined as clinical T₃ or higher or metastatic disease at time of diagnosis⁶². This, however, needs further validation before possible implementation into clinical practice.

Risk calculators

Risk calculators including several predictive factors to stratify patients for prostate biopsy have been developed. Two well known calculators that are online available are the PCPT and the ERSPC risk calculator^{63,64}. The first includes serum PSA, DRE results, age, family history of prostate cancer, ethnicity and prior biopsy. The latter includes serum PSA, DRE results, TRUS findings, prior biopsy and prostate volume. The use of risk calculators allows a more individual assessment of prostate cancer risk and provides a better predictive accuracy compared to PSA alone⁶⁵.

PSA derivatives

PSA derivatives have been evaluated in the attempt to enhance the diagnostic accuracy of total PSA: age-specific total PSA cutoffs, total PSA density, total PSA velocity and total PSA doubling time. Age-specific PSA cutoff values were suggested to enhance the predictive value of PSA. The suggested cutoff values were: 40-49 years old: 2.5 ng/ml, 50-59: 3.5 ng/ml, 60-69: 4.5 ng/ml and 70-79: 6.5 ng/ml. However, the use of an age-specific total PSA cutoff is not validated and criticized for missing clinically significant cancers in older men⁶⁶.

PSA density is defined as the total serum PSA level divided by the volume of the prostate (in grams). A PSA density of 0.15 ng/nl/g or higher has been considered abnormal and

suspicious for cancer. However, the value of this test remains controversial⁶⁷. PSA density correlated with biopsy outcome, tumour aggressiveness and unfavourable pathological features in several studies⁶⁸⁻⁷⁰. However, other studies could not validate these results^{71,72}. In addition, PSA density requires transrectal ultrasound, which is time-consuming, expensive and causes patient discomfort. All together, PSA density is not widely used in clinical practice.

PSA dynamics have been extensively studied for their assumed predictive value to discriminate between benign and malign conditions of the prostate. This includes PSA velocity, the change in PSA over time, and PSA doubling time, the number of months for a certain level of PSA to increase by a factor of two. PSA dynamics are indisputably correlated with the diagnosis of prostate cancer on biopsy. However, there is no sufficient evidence that PSA velocity or PSA doubling time has additional diagnostic value beyond the use of total PSA. Thus, there is no justification for the use of PSA dynamics in clinical decision making before treatment in early-stage prostate cancer⁷³. PSA dynamics are however valuable to monitor treatment. Recurrence after radical prostatectomy can be monitored with high sensitivity using PSA doubling time. Although currently widely used, PSA response to chemotherapy in castrate-resistant prostate cancer patients does not predict long term benefit adequately.

PSA molecular forms

PSA circulates in blood either in a stable complexed form or in an unbound 'free' form. Complexed PSA is bound to proteins: α_1 -antichymotrypsin (ACT), α_2 -macroglobulin (A2M) and α_1 -protease inhibitor (API). A lower percent free PSA (free PSA/total PSA \times 100) is correlated with a higher probability of finding prostate cancer on biopsy^{74,75}. The use of percent free PSA has been approved as a diagnostic marker by the Food and Drug Administration in men with PSA levels 4.0-10.0ng/ml. A cutoff value of 25% is generally used. Note that free PSA is less stable than complexed PSA, causing greater analytic variability. Suboptimal blood sample handling can considerably influence free PSA levels⁷⁶.

Free PSA exists in different molecular isoforms, including pro-PSA, BPH-associated BPSA and intact free PSA^{77,78}. Several studies report significantly higher levels of pro-PSA in patients with prostate cancer, and decreased levels of BPSA and intact free PSA⁷⁹⁻⁸¹. This implies that pro-PSA might be a purer biomarker for prostate cancer than free-PSA. Pro-PSA has also been suggested to selectively identify patients with more aggressive prostate cancer. Its suggested additional diagnostic and prognostic value has yet to be validated.

Human kallikrein 2 (hK2) and Urokinase Plasminogen Activation (uPA) are potential future prostate cancer biomarkers that are thus far not validated. HK2 is from the same gene family as PSA, but differ in their enzymatic activity⁸². Several studies have shown that the use of a combination of hK2 with free and total PSA might improve the predictive value for prostate cancer^{83,84}. HK2 might also have prognostic value^{85,86}. The serum protease uPA might be involved in tumour development and progression through degradation of the extra-cellular matrix⁸⁷. The potential role of uPA as a biomarker of metastatic prostate cancer needs to be validated in large multicenter studies.

MicroRNAs

The discovery of microRNAs (miRNA) in 2004 was a revolutionary step in understanding the mechanisms regulating gene expression and function^{88,89}. Subsequently, it was reported that miRNAs play an important role in cancer by initiating carcinogenesis and driving progression⁹⁰.

MiRNAs are small endogenous non-coding RNAs, up to 22 nucleotides long, that regulate gene expression post-transcriptionally. MiRNAs bind to complementary sequences within messenger RNAs (mRNA) to alter their translation by inhibiting their translation or inducing the cleavage of specific target mRNAs⁹¹. In most cases, miRNAs ‘fine-tune’ protein expression (only a modest reduction of the target mRNA concentration)⁹². Occasionally, it causes upregulation or complete destruction of the target mRNA⁹²⁻⁹⁴.

MiRNAs are known to regulate common cellular targeted pathways (intracellular signaling, DNA repair and cellular adhesion/migration)⁹⁵⁻⁹⁷, androgen signaling⁹⁸⁻¹⁰⁰ and apoptosis avoidance^{101,102}. The exact role of miRNAs in the development and progression of prostate cancer is still being investigated. Yet, miRNAs are promising potential biomarkers and novel therapeutic targets for prostate cancer.

Circulating tumour cells

The importance of circulating tumour cells (CTC) was already acknowledged in 1869 by Thomas Ashworth, an Australian physician who observed CTCs microscopically¹⁰³. Only recent advances in technology facilitate a reliable method for the detection of CTC in blood. The presence of CTCs in blood proved to be associated with overall survival in patients with metastatic breast^{104,105}, colorectal^{106,107} and prostate cancer^{108,109}.

In castrate resistant prostate cancer (CRPC), CTC number before and after treatment is an independent predictor of survival. This is a strong predictor both as a continuous

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variable and when using discrete cutoff values (≥ 5 CTC/7.5ml of blood vs. < 5 CTC)¹⁰⁸⁻¹¹⁰. Post-treatment CTC number showed to be a stronger prognostic factor for survival than a 50% decline in PSA (AUC 0.87 vs. 0.62). CTCs are approved by the Food and Drug Administration as a prognostic biomarker to monitor disease status in patients with metastatic breast, colorectal and prostate cancer. To further explore the potential link to survival, CTCs have been incorporated as an exploratory end point in several phase II and III trials¹¹¹.

URINE MARKERS

PCA3

In 1999, Bussemakers et al. first identified and characterized the differential display clone 3 (DD3, later called PCA3) gene, to date one of the most prostate cancer specific genes¹¹². PCA3 is non-coding RNA and located on chromosome 9q21-22. Its function is unknown. PCA3 is highly overexpressed in prostate tumours compared to adjacent benign prostate tissues, on average between 70 and 80-fold. An upregulation is seen in 95% of the primary prostate tumours and no PCA3 expression is found in non-prostate tissue (i.e. benign and malign tissue from breast, cervix, endometrium, ovary and testis; cell lines originating from bladder, kidney and ovarian cancer)¹¹².

In the initial PCA3 studies, a real-time RT-PCR analysis was used for the quantification of PCA3 messenger RNA (mRNA) in prostate tissue. Later, Hessels et al. developed a dual time resolved fluorescence (TRF)-based RT-PCR assay to detect PCA3 mRNA in urinary sediments after digital rectal examination (DRE)¹¹³. A urine test provides a non-invasive method to obtain prostate (cancer) cells, which makes it suitable for clinical purposes. A DRE is performed to mobilize prostatic cells towards the prostatic urethra, which are flushed out with the first voided urine. A prostate massage is obsolete and causes needless patient discomfort, as a regular DRE sheds enough cells into urine for analysis. In 2006, the ProgenSA PCA3 test was introduced, a transcription-mediated amplification (TMA) assay¹¹⁴. This assay is also performed on first voided urine samples after DRE, but it is a simpler, faster and sensitive enough method compared to the initial RT-PCR based assay; therefore, more viable for widespread clinical implementation. The PCA3 score is the ratio of PCA3:PSA mRNAs multiplied by 1000. The ProgenSA PCA3 test is commercially available and Conformité Européenne (CE)-approved since November 2006 to aid in the decision to take initial or repeat biopsies. The Food and Drug Administration approval process is currently ongoing.

The clinical utility of PCA3 and its additional predictive value beyond PSA has been extensively studied. PCA3 has been validated as a reliable predictor of prostate cancer at initial or repeat biopsy^{113,115-118}. Currently, a cutoff value of 35 is used, resulting in a sensitivity of 47-69% and a specificity of 72-79%^{114,116-118}. However, the optimal cutoff value is subject to debate. Several studies indicate that a cutoff value of 20 or 25 might be preferable, missing less prostate cancers and still preventing a considerable amount of prostate biopsies¹¹⁵. Future studies will have to clarify this issue. Furthermore, PCA3 showed to be an independent predictor of prostate cancer in addition to established prostate cancer risk factors (age, PSA, DRE, prostate volume and biopsy history)^{119,120}. The use of PCA3-based nomograms has recently been validated¹²¹, providing a novel tool for clinical decision making.

It was hypothesized that PCA3 might be associated with more aggressive cancer. This was based on the theory that aggressive prostate cancer cells are more invasive and would therefore more easily shed into the prostatic ductal system after DRE¹²². However, to date, the prognostic value of PCA3 is considered to be limited. Some studies found a correlation of PCA3 with Gleason score^{115,117,123}, but this is contradicted by a range of other studies that show no (additional) predictive value for Gleason score^{122,124-126}. As concluded by Auprich et al., the clinical value of PCA3 to predict aggressive prostate cancer at radical prostatectomy seems to be marginal at best¹²⁴. PCA3 has been shown, however, as a valuable predictor of tumour volume and insignificance of prostate cancer^{117,124,126}. Data on predictive value for extracapsular extension are conflicting^{117,124,127}. Furthermore, PCA3 currently has no role in risk assessment during active surveillance protocols, though this requires further investigation in larger studies^{126,128}.

TMPRSS2-ERG

For a complete description of the gene fusion TMPRSS2-ERG, see section tissue markers. In summary, TMPRSS2-ERG is a fusion of TMPRSS2 (the androgen-regulated trans-membrane protease, serine 2) to Ets family genes (oncogenic transcription factors). Oncogene ERG is the most commonly involved Ets family member in gene fusion. It occurs in approximately half of Caucasian prostate cancer patients.

A publication in 2006 showed the feasibility to detect TMPRSS2-ERG fusion transcripts non-invasively in urinary sediments obtained after DRE using an RT-PCR-based research assay¹²⁹. Since then, extensive research has been performed on the clinical applicability of this urine test. A sensitivity of 37% and specificity of 93% to predict prostate cancer was reported, resulting in a positive predictive value of 94%¹³⁰. Although not (yet)

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validated, this test is assumed to improve the specificity of established prostate cancer risk calculators.

Urine marker panel

Given the tumour heterogeneity in prostate cancer, the use of a panel of biomarkers may provide the best diagnostic accuracy. Hessels et al. evaluated the combination of PCA3 with TMPRSS2-ERG fusion transcripts detected in the urine, showing an improved sensitivity of 73%, compared to 62% for PCA3 alone, without compromising the specificity for detecting prostate cancer¹³⁰. A recent study confirmed an enhanced predictive value of PCA3 combined with TMPRSS2-ERG¹³¹. In conclusion, these preliminary results on the combined use of PCA3 and TMPRSS2-ERG seem promising but require further validation. Future studies will have to assess the use of other (novel) biomarker panels.

So, many new biomarkers are ready for ‘prime time’, yet it needs carefully designed studies to test the exact clinical positioning. In the clinical arena two main themes can be discriminated. Develop methods to better predict biopsy outcome; once the decision to take a biopsy has been taken, the man is a patient; a patient with or without prostate cancer. This is a tough challenge since the man with indolent cancer should not be bothered with a biopsy, yet the ones in the low PSA ranges with aggressive disease should be identified. Once the cancer is diagnosed we should better predict the prognosis and therapy need/response. This will require significant efforts from molecular pathology.

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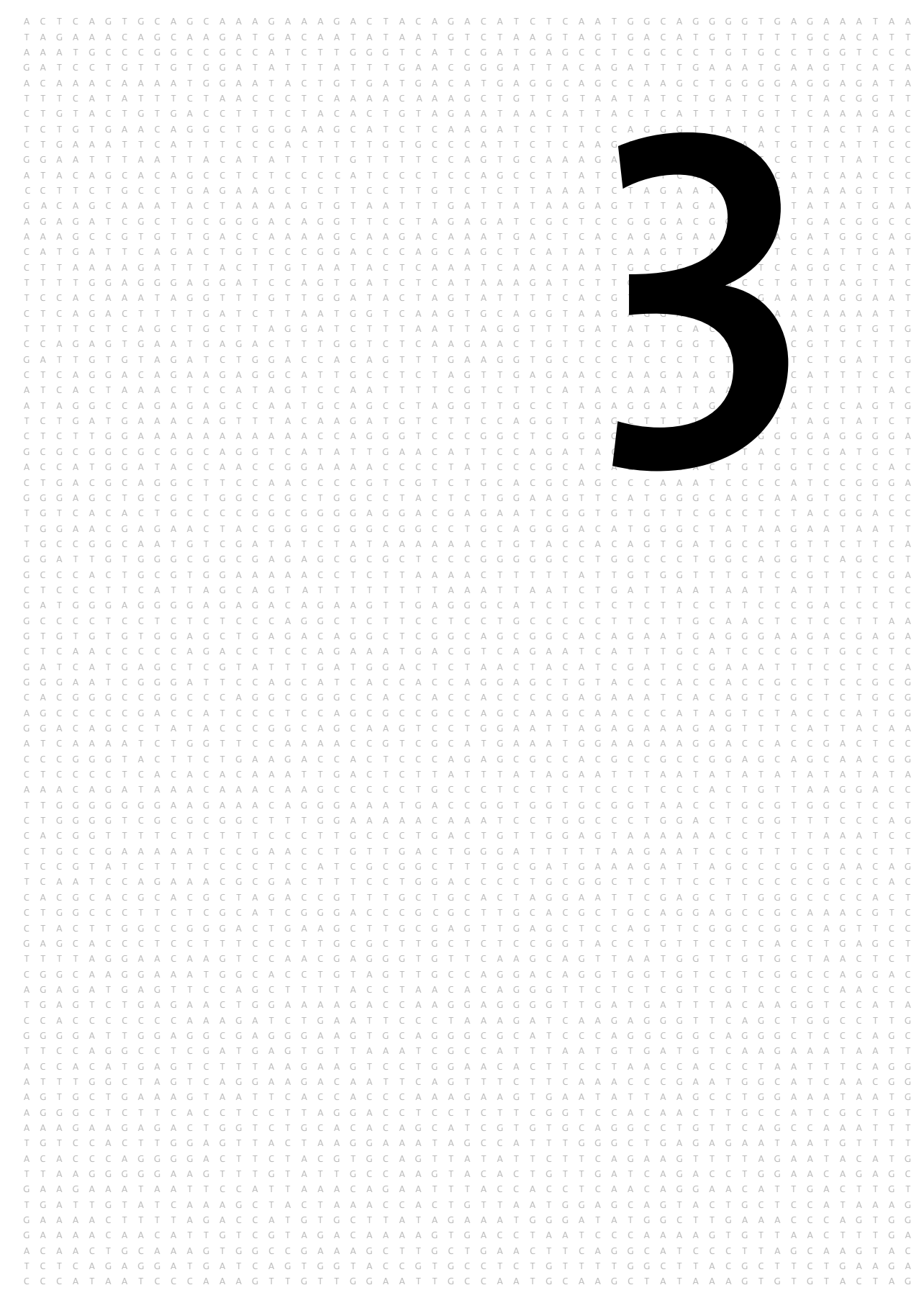
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Value of PCA3 to predict biopsy outcome, and its potential role in selecting patients for multiparametric MRI

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Published in: Int J Mol Sci. 2013 May 28;14 (6):11347-55

ABSTRACT

PCA3 (Prostate CAncer gene 3) and multiparametric 3 tesla MRI are new promising diagnostic tools in the detection of prostate cancer. Our aim was to study the clinical value of the ProgenSA PCA3-test: its predictive value for biopsy outcome, Gleason score and MRI outcome. We evaluated retrospectively 591 patients who underwent a ProgenSA PCA3-test at the Radboud University Nijmegen Medical Centre between May 2006 and December 2009. Prostate biopsies were performed in 290 patients, a multiparametric 3 tesla MRI of the prostate was performed in 163/591 patients. The PCA3-score was correlated to biopsy results and MRI outcome. The results show that PCA3 was highly predictive for biopsy outcome ($p < 0.001$), there was no correlation with Gleason score upon biopsy ($p = 0.194$). The PCA3-score of patients with a suspicious region for prostate cancer on MRI was significantly higher ($p < 0.001$) than in patients with no suspicious region on MRI (52 vs. 21). In conclusion, PCA3 is a valuable diagnostic biomarker for prostate cancer, it did not correlate with Gleason score. Furthermore, multiparametric MRI outcome was significantly correlated with the PCA3-score. Thus PCA3 could be used to select patients that require MRI. However, in patients with a negative PCA3 and high clinical suspicion of prostate cancer also a multiparametric MRI should be done.

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INTRODUCTION

Prostate cancer is the second most commonly diagnosed cancer in men, accounting for 14% of all cancers in males¹. Since the routine use of serum prostate-specific antigen (PSA) testing, prostate cancer detection has increased considerably. In 2011 an estimated 903,500 men will be diagnosed with prostate cancer worldwide, compared to 679,000 men in 2002^{1,2}. However, one of the main drawbacks of PSA testing is the lack of specificity, resulting in a high negative biopsy rate of 60-75%³. Novel diagnostic approaches are required to improve our ability to detect prostate cancer and to identify better which patients require (repeat) prostate biopsy. PCA3 (*Prostate CAnker gene 3*) and 3 tesla multiparametric magnetic resonance imaging (MRI) are new promising diagnostic tools in the detection of prostate cancer.

PCA3 is currently the most specific prostate cancer gene. Since its identification in 1999, extensive research has been performed to assess the clinical utility of PCA3 in the diagnosis of prostate cancer⁴. The current indication to use the PCA3-test is to aid in the decision to perform repeat biopsies. A cutoff value of 35 is used, as this gives an optimal balance between sensitivity (47-69%) and specificity (72-79%) to predict prostate cancer in repeat biopsies⁵⁻⁸. In contrast, the specificity of a PSA level between 4.0-10.0 ng/ml to predict prostate cancer in repeat biopsies is 25-40%³. A recent study provided evidence that the PCA3-test could also be useful to aid in the decision to perform initial biopsies⁹. They suggested that a cutoff value of 20 might be optimal for men with no previous biopsies and a PSA level of 2.5-10.0ng/ml. This would avoid 40% of biopsies; 95% of men with a Gleason ≥ 7 prostate cancer had a PCA3-score ≥ 20 .

Concurrently, multiparametric MRI is being used increasingly in the diagnostic process of prostate cancer. This MRI examination consists of anatomic (T2W) images, dynamic contrast-enhanced (DCE) MRI, diffusion weighted imaging (DWI) and proton MR spectroscopic imaging (MRSI). These techniques have a high accuracy (80-90%) to detect prostate cancer in the gland¹⁰⁻¹³, if read by an experienced radiologist. A recent study showed promising results for discrimination among different aggressiveness classes¹⁴. However, due to additional expenses and limited availability compared to the conventional transrectal ultrasound (TRUS)-guided biopsy, the routine application of MR-guided biopsy is not yet feasible. An additional, cheaper and more practical test to select patients who require multiparametric MRI, and potentially subsequent MR-guided biopsy, would be valuable.

Our aim was to study the value of the clinical use of the ProgenSA PCA3-test. We evaluated PCA3 for its predictive value for biopsy outcome, Gleason score and multiparametric MRI outcome. To our knowledge, the potential correlation between PCA3 and MRI outcome has never been evaluated before, and is important in the light of the increasing role of MRI in the diagnosis of prostate cancer.

MATERIAL AND METHODS

Study design

All 591 patients who underwent a ProgenSA PCA3-test in the Urology outpatient clinic of the Radboud University Nijmegen Medical Centre between May 2006 and December 2009 were evaluated retrospectively. Urine samples were collected and ProgenSA PCA3-tests performed as described by Groskopf et al⁶. The indication to perform a PCA3 test was an elevated PSA level, a family history of prostate cancer or previous negative prostate biopsies. Patient characteristics, number of previous biopsies, biopsy results, MRI outcome and follow-up results were documented. Prostate biopsies were performed within six months after the PCA3-test in 290 patients, using a 10 cores biopsy regimen. Prostate biopsies were performed based on clinical grounds: PSA level, DRE (digital rectal examination) findings, family history and PCA3 score. A 3 tesla multiparametric MRI of the prostate was performed in 115/290 patients that also underwent prostate biopsies (40%).

MRI was performed using a 3 tesla MR scanner (Siemens Trio® Tim). The multiparametric MRI consisted of a combination of anatomical T2-weighted T2W images with the following functional imaging modalities DWI, DCE MRI and MRSI^{15,16}. MRI was read by radiologists experienced in multiparametric MRI. Based on their MRI reports, MRI outcomes were categorized into two groups: no suspicious region for prostate cancer or an evident suspicious region for prostate cancer.

Statistical analysis

The predictive value of PCA3 for biopsy outcome, Gleason score and MRI outcome was studied. Marker values were log-transformed to obtain a normal distribution. The Independent Sample T-test and the non-parametric Mann-Whitney U test were used to assess significance levels. Two-sided P values of 0.05 or less were considered to indicate statistical significance. Statistical analyses were performed using Statistical Package for Social Sciences (SPSS, Chicago, USA) version 16.0 for Windows.

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RESULTS

The value of PCA3 to predict biopsy outcome

In total, 591 patients that had a ProgenSA PCA3-test were evaluated in this study. Patients characteristics and biopsy results are shown in table 1. The indication for a ProgenSA PCA3 test was an elevated PSA level. Prostate biopsies were performed within six months after the PCA3-test in 290/591 patients. prostate cancer was found in 41% of these biopsies. PCA3 was highly predictive for biopsy outcome, both when using PCA3 cutoff value 35 ($p<0.001$) and 20 ($p<0.001$). PCA3 was elevated (≥ 35) in 222/591 patients, of which 162 patients underwent biopsies. In total, 89/162 patients were diagnosed with prostate cancer, leading to a positive predictive value (PPV) of the PCA3-test of 55%. Of the 369 patients with a PCA3-score <35 , 128 underwent biopsies of which 30 patients (23%) were diagnosed with prostate cancer.

Table 1 Patient characteristics and biopsy results

	Total cohort (n=591)		Biopsies taken* after PCA3 (n=290)	No biopsy taken after PCA3 (n=301)	P value
Age (median, yrs)	64		65	63	0.0230 ^a
DRE suspicious	117		90	27	<0.0001 ^b
Prostate volume (median, cc)	45		49	40	0.0267 ^a
No previous biopsies	255		167	88	<0.0001 ^b
PSA (median, ng/ml)	7.2		7.5	7.1	0.0432 ^a
PCA3 (median)	24		41	17	<0.0001 ^a
	PCA3<35 (n=369)	PCA3 ≥35 (n=222)			
Biopsy taken*	128	162			
Prostate cancer upon biopsy	30	89			

* Biopsy taken within six months after the PCA3 test

Table 2 PSA and PCA3 characteristics of patients who underwent biopsies within six months after the PCA3-test, sorted by biopsy outcome

	No prostate cancer in biopsies (n=148)	HG-PIN in biopsies (n=23)	Gleason ≤ 6 prostate cancer in biopsies (n=67)	Gleason ≥ 7 prostate cancer in biopsies (n=52)
Age median (yrs)	63	65	65	67
PSA median (ng/ml)	6.6	6.3	7.9	14.1
PCA3 median	24	34	56	68
PCA3 ≥ 20	58%	78%	85%	92%
PCA3 ≥ 35	42%	48%	75%	75%

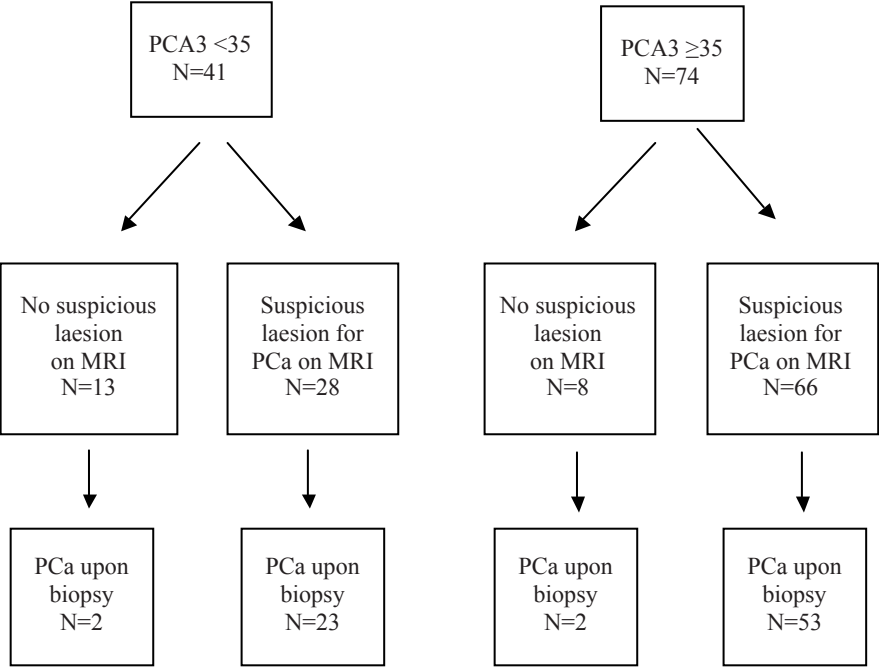
PSA = prostate-specific antigen; PCA3 = Prostate CAncer gene 3;
HG-PIN = high-grade prostatic intraepithelial neoplasia

PSA and PCA3 characteristics are documented in table 2, sorted by biopsy outcome. The median PCA3-score was higher in patients with a Gleason score ≥ 7 upon biopsy, compared to a Gleason score ≤ 6 , but this difference was not statistically significant (68 vs. 56; $p=0.194$). Almost all patients (92%) with a Gleason score ≥ 7 upon biopsy had a PCA3-score ≥ 20 .

The value of PCA3 to predict multiparametric MRI outcome

A total of 115/290 patients that had a PCA3-test and prostate biopsies also underwent a multiparametric 3 tesla MRI of the prostate within 12 months (median 1 month). The median follow up was 24 months. The indication for performing MRI was set on clinical grounds which had not been specifically documented. Patients with a suspicious region for prostate cancer on MRI had a significantly higher PCA3-score than patients with no suspicious region (median 29 vs. 54, $p=0.002$). The PSA level was not significantly different per MRI outcome (median 9.7 vs. 9.5, $p=0.650$). Figure 1 shows a flow diagram with PCA3 score, MRI outcome and biopsy outcome. The majority of patients with PCA3 scores ≥ 35 had a suspicious region for prostate cancer upon MRI and were diagnosed with prostate cancer upon biopsy. In patients with PCA3 scores < 35 and prostate cancer upon biopsy, almost all (23/25 patients) had a suspicious region for prostate cancer upon MRI. Only two patients with PCA3 scores < 35 (PCA3 scores 2 and 7) and no suspicious lesion upon MRI were diagnosed with prostate cancer upon biopsy.

Figure 1 Flow diagram of the 115 patients that had a PCA3 test, multiparametric MRI and prostate biopsies



* PCa = prostate cancer

DISCUSSION

In this study, the PCA3-score was significantly higher in patients with a suspicious region for prostate cancer on MRI compared to patients with no suspicious region. To our knowledge, this is the first report to show a correlation between PCA3 and MRI outcome. There is one previous study in which PCA3 and MRI are combined¹⁷. However, they evaluated whether the potential value of the PCA3 test as a biomarker for prostate cancer diagnosis could be improved by the use of multiparametric MRI for redirecting (TRUS-guided) prostate biopsy. They did not correlate PCA3 score to MRI outcome, therefore, we cannot compare our results to the results of Sciarra et al. Our findings are particularly important in the light of the increasing role of MRI in the diagnosis of prostate cancer. Furthermore, we show that PCA3 was highly predictive for biopsy outcome, but was not significantly correlated with Gleason score upon biopsy.

Our study failed to show a correlation between PCA3 and Gleason score upon biopsy. The PCA3 score was somewhat higher in patients with a Gleason score ≥ 7 prostate cancer compared to patients with a Gleason score ≤ 6 prostate cancer (68 vs. 56), but the difference was not significant ($p=0.194$). Recently, Auprich and colleagues concluded that the PCA3 score is low in indolent cancers, but that within the group of significant cancers this marker did not further differentiate¹⁸.

Several studies have been performed to evaluate the potential prognostic value of PCA3. Some studies documented a correlation of PCA3 with the Gleason score^{7,9,19}. However, a range of studies reported no (additional) value of PCA3 to predict aggressive disease^{18,20-22}. Our results are in agreement with Auprich et al, i.e. that the prognostic value of PCA3 is limited to discriminate indolent from significant cancer. Due to the low number ($n=10$) of cases with indolent cancer in our study (according to the Epstein criteria: stage T1c, Gleason score ≤ 6 , PSA density ≤ 0.15 and $\leq 33\%$ positive cores upon biopsy) we cannot unequivocally support Auprich.

Even though PCA3 is a continuous variable, a PCA3 cutoff value of 35 is often used. However, the use of PCA3 cutoff value is dependent on the indication. The study that led to FDA approval was based on a high negative predictive value (NPV) (missing less prostate cancers and still preventing a considerable amount of prostate biopsies), which was achieved at a cut off of 20 (NPV 88%). In our study, the diagnostic value of PCA3 for predicting biopsy outcome was comparable when using a cutoff value 20 or 35. However, of patients with a Gleason score ≥ 7 upon biopsy, 25% had a PCA3 score < 35 and only 8% had a PCA3 score < 20 . These results correspond well to the results of de la Taille et al⁹, contributing to the evidence that a PCA3 cutoff value of 20 might be preferable.

Multiparametric 3 tesla MRI is effective to detect and localize clinically significant prostate cancer (Example is shown in Fig. 2)^{5,16}. In this retrospective study, we analysed a subcohort of patients that underwent PCA3, multiparametric MRI and prostate biopsies. We show that PCA3 score is correlated with MRI outcome. The majority of patients with an elevated PCA3 score had a suspicious lesion upon MRI and was diagnosed with prostate cancer upon biopsies. Thus, PCA3, in addition to biochemical and clinical parameters, could be used to select patients that require MRI. However, due to the selection bias in this retrospective, observational study, our study is purely hypothesis generating and the clinical applicability of PCA3 to select patients that require MRI must be evaluated in prospective studies for its sensitivity, specificity, PPV and NPV. Also, the PCA3 cutoff value that will give an optimal balance between sensitivity and specificity to predict

MRI outcome should be evaluated prospectively. In this study, using a PCA3 with cutoff score 20 did not change our results significantly (data not shown). Furthermore, we show that MRI might be of additional value in the subgroup of patients with a low PCA3 score and a high clinical suspicion of prostate cancer. As 23/28 patients with low PCA3 scores and a suspicious lesion upon MRI were diagnosed with prostate cancer. The NPV of the combined use of PCA3 and MRI was very high as only two patients with low PCA3 scores and no suspicious lesion upon MRI, were diagnosed with prostate cancer (Gleason 6 and 8).

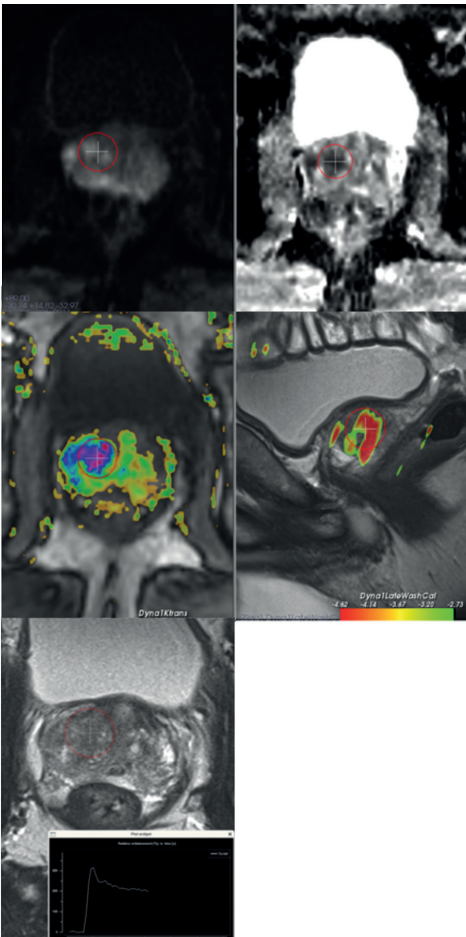


Figure 2 Multiparametric magnetic resonance (MR) imaging of the prostate of a 77 year old male, PSA level of 53, Prostate CAncer gene 3 (PCA3) score of 43 and 2x negative transrectal ultrasound-guided biopsy sessions. Upper left image: axial diffusion weight imaging (DWI-b 1400) shows high signal; this combined with low signal on upper right image: axial apparent diffusion coefficient (ADC) map, indicates restriction and thus is suspicious for intermediate grade cancer. Middle left: axial, and middle right: sagittal dynamic contrast-enhanced MR images show increased vascular permeability. The curve (bottom) has a steep rise and wash out which indicates cancer. Bottom image: axial T2 weighted MR image shows homogeneous low signal which also fits tumour. MR-guided biopsy revealed in all 3 cores 75% Gleason 4+3 prostate cancer.

By demonstrating a correlation between PCA3 and MRI outcome, our study is the first step in the assessment of the clinical applicability of the combined use of PCA3 and MRI in the diagnostic process of prostate cancer. Based on our results, we hereby propose a prospective study design to further assess the hypothesis that PCA3 could select patients that require MRI. Ideally, all men in the prospective study would undergo a PCA3-test, multiparametric MRI, and prostate biopsies. This would, however, lead to practical and financial objections. Therefore, we propose a prospective study in which men scheduled for TRUS-guided biopsies and PSA < 10 ng/ml will be randomized based on their PCA3-score. Men with a PCA3-score <20 will undergo TRUS-guided biopsies; men with a PCA3-score ≥ 20 will undergo MRI and subsequent biopsies if suspicious lesion(s) are seen on MRI. We suggest a PCA3 cutoff score of 20 as this has shown to detect the majority of men with clinically significant prostate cancer.

CONCLUSIONS

PCA3 is a valuable diagnostic biomarker for prostate cancer, it did not correlate with biopsy Gleason score. Furthermore, there was a significant correlation of PCA3 with multiparametric MRI outcome. This generates the hypothesis that PCA3 could be used to select patients that require MRI. However, in patients with a negative PCA3 and high clinical suspicion of prostate cancer, also a multiparametric MRI should be done. Based on our results, we propose a prospective study design to evaluate the clinical applicability of this hypothesis.

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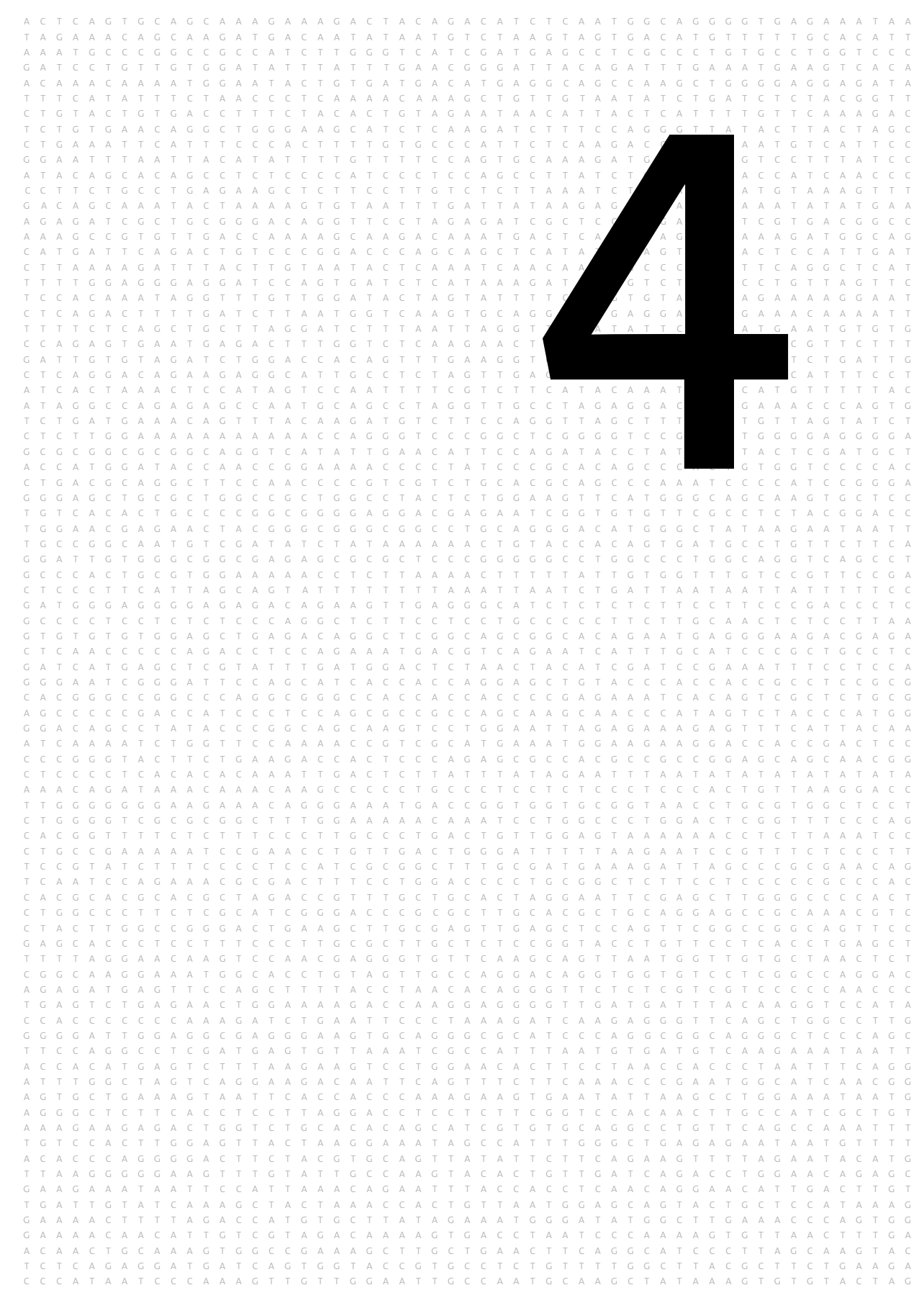
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Prospective multicentre evaluation of PCA3 and TMPRSS2-ERG gene fusions as diagnostic and prognostic urinary biomarkers for prostate cancer

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ABSTRACT

Introduction

PCA3 and TMPRSS2:ERG gene fusions are promising prostate cancer specific biomarkers that can be measured in urine. The objective was to evaluate the diagnostic and prognostic value of ProgenSA PCA3 and TMPRSS2-ERG gene fusions (as individual biomarkers and as a panel) for prostate cancer in a prospective multicentre setting.

Material and Methods

At six centres, post-DRE first-catch urine specimens prior to prostate biopsies were prospectively collected from 497 men. We assessed the predictive value of ProgenSA PCA3 and TMPRSS2-ERG (quantitative nucleic acid amplification assay to detect TMPRSS2-ERG mRNA) for prostate cancer, Gleason score, clinical tumour stage, and prostate cancer significance (individually and as a marker panel). This was compared to serum PSA and the ERSPC risk calculator. In a subgroup (n=61) we evaluated biomarker association with prostatectomy outcome. Univariate and multivariate logistic regression analysis and ROC (Receiver Operating Curves) were used.

Results

Urine samples of 443 men contained sufficient mRNA for marker analysis. Prostate cancer was diagnosed in 196/443 men. Both PCA3 and TMPRSS2-ERG had significant additional predictive value to the ERSPC risk calculator parameters in multivariate analysis ($p < 0.001$ and resp. $p = 0.002$). The AUC increased from 0.799 (ERSPC risk calculator), to 0.833 (ERSPC RC+PCA3), to 0.842 (ERSPC RC+PCA3+TMPRSS2-ERG) to predict prostate cancer. Sensitivity of PCA3 increased from 68% to 76% when combined with TMPRSS2-ERG. TMPRSS2-ERG had significant additional predictive value to the ERSPC risk calculator to predict biopsy Gleason score ($p < 0.001$) and clinical tumour stage ($p = 0.023$), whereas PCA3 did not.

Conclusions

TMPRSS2-ERG had independent additional predictive value to PCA3 and the ERSPC risk calculator parameters for predicting prostate cancer. Furthermore, TMPRSS2-ERG had prognostic value, whereas PCA3 did not. By implementing the novel urinary biomarker panel PCA3 and TMPRSS2-ERG into clinical practice, this would lead to a considerable reduction of the number of prostate biopsies.

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INTRODUCTION

The gold standard for the diagnosis of prostate cancer is based on the histopathological evaluation of prostate biopsies, an invasive procedure with significant morbidity. Since localized prostate cancer often does not present with symptoms, the selection of men qualifying for prostate biopsies relies on serum PSA (prostate-specific antigen) testing and digital rectal examination (DRE). PSA is currently the only widely used serum biomarker for prostate cancer. However, PSA has a low specificity of 25-40% in the so-called 'grey area' of PSA levels 4.0-10.0 ng/mL, resulting in a high negative biopsy rate^{1,2}. Moreover, widespread PSA testing leads to the diagnosis of clinically insignificant tumours, resulting in potential overtreatment, causing morbidity and leading to unnecessary increased health care costs. In the ongoing search for more specific biomarkers for prostate cancer, PCA3 and TMPRSS2-ERG gene fusion transcripts have been identified as promising urinary novel biomarkers^{3,4}.

The ProgenSA PCA3 test is FDA-approved and commercially available to aid in the decision of taking repeat prostate biopsies. A PCA3 cutoff score of 35 is generally used. However, most recent studies show that a lower cutoff score of 25 might be preferable⁵⁻⁸. The results of a possible correlation with established prognostic factors (histologic Gleason grade and tumour stage) are conflicting^{5,8-11}. The consensus in most papers is that PCA3 is often negative in patients with indolent cancer, yet in the clinically significant cancers there is no evidence for an association with histo-pathological prognostic factors (stage, grade).

In prostate cancer, TMPRSS2 (androgen-regulated trans-membrane protease, serine 2) can be fused to ERG (member of the ETS family of oncogenes)⁴. These TMPRSS2-ERG gene fusions are highly specific for prostate cancer and are present in approximately half of the Caucasian prostate cancer patients¹². PCA3 and TMPRSS2-ERG are also occasionally found in HG-PIN, in prostate glands in which mostly also prostate cancer is found^[4]. In 2006, the TMPRSS2-ERG gene fusion transcripts were successfully detected in urine samples¹³. This urine test had a sensitivity of 37% and specificity of 93% for the prediction of prostate cancer upon prostate biopsy¹⁴. The prognostic value of this urine test has not been assessed yet.

Considering the heterogeneous character of the disease, the preferred approach in the diagnostic process of prostate cancer will likely be the use of a panel of biomarkers. In 2007, it was shown that the combined use of PCA3 and TMPRSS2-ERG gene fusion transcripts improved sensitivity significantly¹⁴. This was recently confirmed by Tomlins et

al., however, that study was not prospectively conducted¹⁵. The aim of this study was to evaluate the diagnostic and prognostic predictive value of ProgenSA PCA3 + TMPRSS2-ERG gene fusions (as individual biomarkers and as a panel) in a prospective multicentre setting, in accordance with the STARD criteria¹⁶.

MATERIAL AND METHODS

Study design - clinical study

Inclusion criteria were: men scheduled for prostate biopsy based on elevated serum PSA levels (≥ 3 ng/ml), family history of prostate cancer, or an abnormal DRE. Exclusion criteria were: history of prostate cancer, medical therapy known to affect serum PSA levels, symptoms of urinary tract infection, prostate biopsy within three months prior to enrolment, or invasive treatment for benign prostatic hyperplasia (BPH) within six months prior to enrolment. Subjects were recruited at six urology centres in the Netherlands (Radboud University Nijmegen Medical Centre, Nijmegen; Academic Medical Centre, Amsterdam; ZGT Hospital, Hengelo; Canisius Wilhelmina Hospital, Nijmegen; Schepers Hospital, Emmen, and St. Elisabeth Hospital, Tilburg). Prostate biopsies were performed and evaluated per hospital's standard procedure (8-12 biopsy cores). One experienced genitourinary pathologist reviewed all biopsy Gleason scores independently, being blinded for the biomarker scores. Three biopsy samples were not available for Gleason review; here the local Gleason scores were used. The respective independent ethics committees approved the study protocol and written informed consent was obtained.

Specimen processing

First-catch urine specimens after DRE were processed using a validated standard operating procedure (SOP) based on the integration of procedures described by Groskopf et al¹⁷, whole urine, and urinary sediments were prepared as described by Hessels et al¹⁸. Shortly, first voided urine after DRE was collected in a coded container with 4 ml 0.5 M EDTA. Immediately, 2.5 ml of the urine was transferred to a PCA3 urine sample collection tube containing sample transport medium (Gen-Probe Inc.). The urine samples were immediately cooled to 4°C and were mailed with cold packs to NovioGendix Research BV (Nijmegen, The Netherlands). Urine samples were processed within 48 h after collection to guarantee good sample quality. The urine, EDTA stabilised, was centrifuged at 4°C and 1.800 x g for 10 minutes. The obtained urinary sediments were washed twice with ice-cold buffered sodium-chloride solution. Upon centrifugation at 4°C and 1.000x g for 10 minutes, the sediments were snap frozen in liquid nitrogen and stored at -70°C. The

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urine in the PCA3 urine sample collection tube was used for the Progenisa PCA3 test at NovioGendix as described by Groskopf et al¹⁷. The PCA3 score was calculated as [PCA3 mRNA]/[PSA mRNA] x 1000.

Quantitative real-time PCR for PCA3 and TMPRSS2-ERG

RNA was extracted from the urinary sediments using a modified Tripure reagent (Roche, Cat no. 11 667 165 001) protocol. After the chloroform extraction, GlycoBlue (Ambion, Cat no. AM 9515) was added to the aqueous phase to precipitate the RNA using isopropanol. The RNA samples that were obtained were DNase treated prior to the amplification protocol using DNase I enzyme (Invitrogen, Cat. No.18068-015). The RNA samples were purified using GlycoBlue and sodium acetate (Ambion, Cat no. AM 9740). Total RNA from the sediments was used to generate amplified sense-strand cDNA using the Whole Transcriptome (WT) Expression Kit (Ambion, Cat no. 4411974) according to the manufacturers protocol. Fluorescence based real-time PCR assays were designed and optimised specific for TMPRSS2-ERG gene fusion transcripts and PSA. The TMPRSS2-ERG assay detects the three prime gene fusions identified in prostate cancer⁴. The primers were located in exon 1 of TMPRSS2 and exon 4 of ERG. The probe was located in exon 4 of ERG and labeled with two fluorochromes; a 5' end reporter fluorescent dye and a quencher dye at the 3' end. The primers and probe for the PSA assay are located in exon 1 and 2 of the PSA gene (Table 1). All primers and probes were designed by and manufactured by TIB Molbiol Berlin. Blunt-ended PCR products were cloned in the pCR®-Blunt cloning vector (Invitrogen). Calibration curves with a wide linear dynamic range (10 – 1.000.000 copies) were generated using serial dilutions of the plasmids. The amplification efficiency of the primer pair was determined using the calibration curve and was >1.85. Control samples with known template concentrations were used as a reference. Two µl of each cDNA sample were amplified in a 20 µl PCR reaction containing 10 pmol of each primer, 2 pmol of TaqMan probe and 1x ProbeMaster mix (Roche). The following amplification conditions were used: 95°C for 10 minutes followed by 50 cycles at 95°C for 10 seconds, 60°C for 30 seconds and cooling at 40°C for 55 seconds (LightCycler LC480, Roche). The crossing point (Cp) values were determined using the Lightcycler 480 SW 1.5 software (Roche). The Cp values of the samples were converted to concentrations by extrapolation in the generated calibration curve. For TMPRSS2-ERG, real-time PCR experiments were performed twice for each sample, and classified as positive for TMPRSS2-ERG whenever they contained at least ≥10 copies in a single experiment. A fluorescent signal < 1(465-510) was classified as background signal. The assay performance of the real-time PCR experiments was evaluated during in-study validation. The reference control samples had an inter- and intra-assay variation < 30%.

Table 1 Primer sequences and TaqMan probes

Gene	GenBank™	Primer	Position	Sequence
TMPRSS2-F	NM_005656	forward	1 - 17	5'- CGC GAG CTA AGC AGG AG-3'
ERG-R	NM_004449	reverse	315 - 334	5'-GTC CAT AGT CGC TGG AGG AG-3'
ERG-probe	NM_004449		80 - 109	5'-FAM-TGG TCC TCA CTC ACA ACT GAT AAG GCT TCC-BBQ-3'
PSA-F	NM_001648	forward	74- 91	5'- CGT GAC GTG GAT TGG TGC-3'
PSA-R	NM_001648	reverse	179 - 194	5'- GCC GCA GAC TGC CCT G-3'
PSA-probe	NM_001648		97 - 122	5'-610-CCC TCA TCC TGT CTC GGA TTG TGG GA-BBQ-3'

GenBank™ = Database accession number

Data collection

The following data were extracted from the records: age, serum PSA, DRE and transrectal ultrasound (TRUS) results, prostate volume, biopsy results (current and history), radiological results, clinical TNM stage (if diagnosed with prostate cancer) and radical prostatectomy results (if applicable). These data and the coded assay results were entered in a secured preset web-based database with audit trail (in compliance with the ICH-GCP guidelines). The assay results and revised Gleason scores were not provided to the clinical sites for patient care.

Statistical analyses

PCA3 was assessed both as a continuous and a dichotomous variable with cutoff score 25 and 35. TMPRSS2-ERG was assessed as a dichotomous variable; all samples with measureable TMPRSS2-ERG (≥ 10 copies TMPRSS2-ERG mRNA) were marked positive. The following endpoints were used: prostate cancer, biopsy Gleason score, clinical tumour stage, prostate cancer significance and in a subgroup prostatectomy histological outcome. Clinical significance of the tumour was assessed according to the Epstein criteria: clinical stage $\geq T2$, Gleason score ≥ 7 , PSAD > 0.15 and $> 33\%$ positive cores. The Fisher's exact test (for dichotomous variables) and the nonparametric Mann Whitney test (for continuous variables) were used to test differences in levels of serum PSA, PCA3, and TMPRSS2-ERG for statistical significance between groups of patients. Univariate logistic regression analysis was used to study the influence of the biomarkers on each endpoint separately. Multivariate logistic regression analysis with selection procedures

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was used to test if PCA3 and TMPRSS2-ERG had independently additional predictive value to the ERSPC risk calculator parameters, on the three outcomes. The ERSPC risk calculator parameters are: serum PSA level ng/ml, DRE normal/abnormal, TRUS normal/abnormal, and prostate volume (cc). In addition, we tested which combination of the biomarker panel PCA3 + TMPRSS2-ERG had the best additional discriminative value to the ERSPC risk calculator parameters. The Odds Ratios (OR) and corresponding 95% Confidence Intervals (CI) of the final model of each outcome are presented. The AUCs (Area Under the Curve) of the ROC (Receiver Operating Characteristic) curves were used to measure the additional predictive discrimination of PCA3 and TMPRSS2-ERG to the ERSPC risk calculator parameters. Two-sided P values of < 0.05 were considered statistical significant. All statistical analyses were performed using SPSS® 18.0 software.

RESULTS

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Urine samples of 497 men were collected between September 2009 and July 2011, of which 54 samples were excluded for containing too much (in)organic precipitate or inadequate amounts of prostate cells (<1000 copies PSA mRNA). Thus, in total, urine samples of 443 men were analysed successfully (89%). Patient characteristics are shown in Table 2. Median serum PSA was 7.4 ng/ml, of which 274 men (62%) with serum PSA level 4.0-10.0 ng/ml and 140 men (32%) with serum PSA level ≥10.0 ng/ml. Of the 95 patients that had previous biopsies, this concerned 1, 2, or ≥3 previous biopsies in respectively 70, 17, and 8 patients.

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Total biopsy cohort

Table 3 shows the biomarker characteristics per biopsy outcome: prostate cancer, Gleason score, clinical tumour stage, and prostate cancer significance. Serum PSA, PCA3 and TMPRSS2-ERG were all significantly higher in patients with prostate cancer compared to patients without prostate cancer. Serum PSA and TMPRSS2-ERG were significantly higher in patients with Gleason scores ≥7 (7.8 vs. 10.7 ng/ml and 7% vs. 35% TMPRSS2-ERG positive resp.) and clinical tumour stage T3-T4 (8.0 vs. 12.3 ng/ml and 16% vs. 36% TMPRSS2-ERG positive resp.). PCA3 scores did not differ significantly between these groups. When evaluating a subcohort of patients with PSA levels <10 ng/ml (n= 303, data not shown), only TMPRSS2-ERG correlated significantly (p=0.016) with Gleason score. In this subcohort of patients, only serum PSA correlated significantly with clinical tumour stage (p=0.002).

Table 2 Patient characteristics

	All evaluable men (n = 443) Median (range) / n(%)
Age (yrs)	65 (44-86)
Serum PSA (ng/ml)	7.4 (0.4-756)
PSAD (ng/ml/ml)	0.15 (0.01-22.6)
Abnormal DRE	126 (28%)
Abnormal TRUS	86 (19%)
Prostate volume (ml)	48 (15-200)
Family history PCa	82 (19%)
Initial biopsy	348 (79%)
Diagnosis PCa	196 (44%)
Gleason score ≥ 7	115 (59%)
Gleason score 7	60 (31%)
Gleason score 8-10	55 (28%)
Clinically significant PCa (Epstein)	177 (40%)
Radical prostatectomy	61 (14%)

PSAD = PSA density (serum PSA/prostate volume); DRE = digital rectal examination
TRUS – transrectal ultrasound; PCa = prostate cancer
Clinically significant prostate cancer according to the Epstein criteria:
clinical stage $\geq T_2$, Gleason score ≥ 7 , PSAD > 0.15 and $> 33\%$ positive cores upon biopsy

Table 4 shows the crude OR (95% CI) of the biomarkers predicting prostate cancer, Gleason score, clinical tumour stage, and prostate cancer significance, respectively, using univariate logistic regression analysis. Regarding prostate cancer, all variables were significant predictors ($p < 0.001$). PCA3 demonstrated the highest accuracy in predicting prostate cancer (AUC 0.720). Both PCA3 (OR: 3.64; 95% CI: 2.19-6.05; $p < 0.001$) and TMPRSS2-ERG (OR: 3.28; 95% CI: 1.57-6.85; $p = 0.002$) had independent additional predictive value to the ERSPC risk calculator parameters for predicting prostate cancer, using multivariate models with selection procedure (Table 5). The ERSPC risk calculator parameters had a predictive accuracy for prostate cancer diagnosis of 0.799, compared to 0.833 when PCA3 was included, and 0.842 when both PCA3 and TMPRSS2-ERG were included (Figure 1).

Table 3 Biomarker characteristics by prostate cancer, Gleason score and clinical tumour stage

	Prostate cancer			Gleason score		Clinical tumour stage			Prostate cancer significance*		
	No (n=247) median (range) / n (%)	Yes (n=196) median (range) / n (%)	P- value	≤6 (n=81) median (range) / n (%)	≥7 (n=115) median (range) / n (%)	P- value	T1-T2 (n=124) median (range) / n (%)	T3-T4 (n=72) median (range) / n (%)	Yes (n=266) median (range) / n (%)	Yes (n=177) median (range) / n (%)	P- value
Age	63 (46-80)	66 (44-86)	0.001 ^a	64 (52-85)	67 (44-86)	0.002 ^a	65 (44-85)	66 (54-86)	64 (46-80)	66 (44-86)	0.002 ^a
Serum PSA (ng/ml)	6.8 (0.4-48)	9.2 (0.6-756)	<0.001 ^a	7.8 (0.6-38)	10.7 (2.3-756)	<0.001 ^a	8.0 (0.6-67.9)	12.3 (5.3-756)	6.7 (0.4-48)	9.6 (2.3-756)	<0.001 ^a
PCA3 score	24 (0-219)	60 (0-522)	<0.001 ^a	59 (2-308)	60 (0-522)	0.552 ^a	60 (2-308)	54 (0-522)	24 (0-308)	60 (0-522)	<0.001 ^a
PCA3 score ≥25	118 (48%)	159 (81%)	<0.001 ^b	64 (79%)	95 (83%)	0.580 ^b	101 (82%)	58 (81%)	131 (49%)	146 (83%)	<0.001 ^b
PCA3 score ≥35	98 (40%)	134 (68%)	<0.001 ^b	55 (68%)	79 (69%)	1.000 ^b	88 (71%)	46 (64%)	111 (42%)	121 (68%)	<0.001 ^b
TMPRSS2-ERG ≥10	15 (6%)	46 (24%)	<0.001 ^b	6 (7%)	40 (35%)	<0.001 ^b	20 (16%)	26 (36%)	18 (7%)	43 (24%)	<0.001 ^b

* Clinically significant prostate cancer according to Epstein criteria: clinical stage ≥T2, Gleason score ≥7, PSAD >0.15 and >33% positive cores.
No prostate cancer significance = no prostate cancer + clinically insignificant prostate cancer
PCA3 score = [copies PCA3 mRNA] / [copies PSA mRNA] x 1000; TMPRSS2-ERG positive = ≥ 10 copies TMPRSS2-ERG mRNA;
^a Mann Whitney test; ^b Fisher's exact test.

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Table 4 The crude Odds Ratio (OR) with 95% Confidence Interval (CI) of the biomarkers for the probability of prostate cancer, Gleason score and clinical tumour stage, respectively, using univariate logistic regression

	Prostate cancer				Gleason score ≥ 7				Clinical tumour stage T3-T4				Clinically significant prostate cancer*			
	OR (95% CI)	P value	AUC (95% CI)	OR (95% CI)	P value	AUC (95% CI)	OR (95% CI)	P value	OR (95% CI)	P value	AUC (95% CI)	OR (95% CI)	P value	AUC (95% CI)	OR (95% CI)	P value
Serum PSA (ng/ ml)	1.08 (1.05-1.12)	<0.001	0.67 (0.62-0.72)	1.07 (1.03-1.12)	0.001	0.68 (0.61-0.75)	1.05 (1.02-1.08)	<0.001	1.05 (1.02-1.08)	<0.001	0.71 (0.64-0.79)	1.10 (1.06-1.14)	<0.001	0.72 (0.67-0.78)		
PCA3 score																
<25	1.00 (reference)			1.00 (reference)			1.00 (reference)		1.00 (reference)			1.00 (reference)				
≥ 25	4.70 (3.04-7.27)	<0.001	0.67 (0.62-0.72)	1.26 (0.61-2.59)	0.527	0.52 (0.44-0.60)	0.94 (0.45-1.98)	0.877	0.94 (0.45-1.98)	0.877	0.50 (0.41-0.58)	4.85 (3.08-7.66)	<0.001	0.64 (0.58-0.69)		
PCA3 score																
<35	1.00 (reference)			1.00 (reference)			1.00 (reference)		1.00 (reference)			1.00 (reference)				
≥ 35	3.29 (2.22-4.88)	<0.001	0.64 (0.59-0.70)	1.04 (0.56-1.91)	0.906	0.50 (0.42-0.59)	0.72 (0.39-1.34)	0.305	0.72 (0.39-1.34)	0.305	0.47 (0.38-0.55)	3.02 (2.02-4.50)	<0.001	0.61 (0.55-0.67)		
TPMRSS2-ERG																
<10	1.00 (reference)			1.00 (reference)			1.00 (reference)		1.00 (reference)			1.00 (reference)				
≥ 10	4.74 (2.56-8.80)	<0.001	0.59 (0.53-0.64)	6.67 (2.67-16.66)	<0.001	0.64 (0.56-0.71)	2.94 (1.49-5.79)	0.002	2.94 (1.49-5.79)	0.002	0.60 (0.52-0.68)	4.42 (2.45-7.97)	<0.001	0.64 (0.58-0.71)		

* Clinically significant prostate cancer according to Epstein criteria: clinical stage $\geq T2$, Gleason score ≥ 7 , PSAD > 0.15 and $> 33\%$ positive cores.

PCA3 score = [copies PCA3 mRNA] / [copies PSA mRNA] $\times 1000$; TMPRSS2-ERG positive = ≥ 10 copies TMPRSS2-ERG mRNA; Gleason score = Gleason score ≤ 6 vs. ≥ 7 ;

Clinical tumour stage = T1-T2 vs. T3-T4; Clinically significant prostate cancer = Clinically significant prostate cancer according to Epstein criteria vs. the rest of the patients

(no prostate cancer + insignificant prostate cancer)

AUC = Area Under the Curve

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Table 5 The adjusted Odds Ratios (OR) with 95% Confidence Intervals (CI) of the biomarker for the probability of prostate cancer, Gleason score and clinical tumour stage, respectively, using multivariate logistic regression

	OR (95% CI)	p value	AUC (95% CI)
Prostate cancer:			
ERSPC parameters			0.799 (0.756-0.841)
Serum PSA	1.08 (1.04-1.12)	<0.001	
Volume	0.98 (0.97-0.99)	<0.001	
DRE abnormal*	2.90 (1.60-5.24)	<0.001	
TRUS abnormal†	2.10 (1.06-4.18)	0.035	
PCA3 score ≥25‡	3.64 (2.19-6.05)	<0.001	0.833 (0.795-0.870)
TMPRSS2-ERG ≥10§	3.28 (1.57-6.85)	0.002	0.842 (0.806-0.878)
Gleason score ≥7:			
ERSPC parameters			0.801 (0.738-0.865)
Serum PSA	1.06 (1.02-1.11)	0.003	
Volume	0.99 (0.97-1.01)	0.205	
DRE abnormal*	7.06 (3.17-15.73)	<0.001	
TRUS abnormal†	1.26 (0.52-3.03)	0.613	
TMPRSS2-ERG ≥10§	7.16 (2.54-20.15)	<0.001	0.840 (0.785-0.895)
Clinical tumour stage T3-T4:			
ERSPC parameters			0.824 (0.764-0.884)
Serum PSA	1.05 (1.02-1.08)	0.002	
Volume	0.98 (0.96-1.00)	0.093	
DRE abnormal*	3.98 (1.84-8.62)	<0.001	
TRUS abnormal†	2.91 (1.35-6.27)	0.006	
TMPRSS2-ERG ≥10§	2.60 (1.14-5.90)	0.023	0.834 (0.776-0.892)

AUC = Area Under the Curve
Reference groups: * DRE normal, † TRUS normal, ‡ PCA3 score <25,
§ TMPRSS2-ERG <10 copies

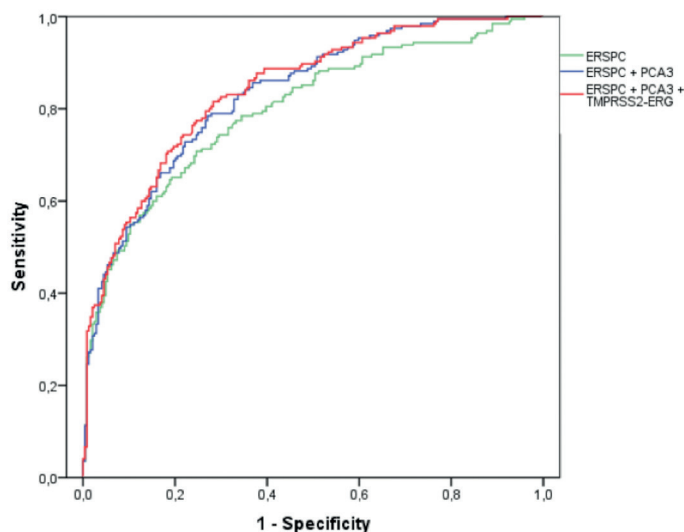


Figure 1 The ROC curves for the three models; 1 using ERSPC risk calculator parameters only (green line, AUC = 0.799), 2 using ERSPC risk calculator parameters and PCA3 (blue line, AUC 0.833), and 3 using ERSPC risk calculator parameters, PCA3, and TMPRSS2-ERG (red line, AUC = 0.842) for the prediction of prostate cancer upon biopsy.

Regarding biopsy Gleason score and clinical tumour stage, serum PSA ($p=0.001$ and $p<0.001$ resp.) and TMPRSS2-ERG ($p<0.001$ and $p=0.002$ resp.) were significant predictors, whereas PCA3 was not, using univariate logistic regression analysis (Table 4). Only TMPRSS2-ERG was an independent predictor of biopsy Gleason score (OR: 7.16; 95% CI: 2.54-20.15; $p<0.001$) and clinical tumour stage (OR: 2.60; 95% CI: 1.14-5.90; $p=0.023$), in addition to the ERSPC risk calculator parameters, using multivariate logistic regression analysis with selection procedure (Table 5).

The urinary biomarker panel was defined negative if having a negative TMPRSS2-ERG (<10 copies mRNA) and PCA3 <25 , and positive if else. It had the best predictive value for prostate cancer compared to all other panels defined by these markers, using multivariate logistic regression models with selection procedure. Therefore, sensitivity and specificity were calculated for this panel, and for PCA3 and TMPRSS2-ERG individually. TMPRSS2-ERG was a highly specific biomarker for clinically significant prostate cancer, with a sensitivity of 24.3% and a specificity of 93.2%. PCA3 (cutoff score 35) had a sensitivity of 68.4% and a specificity of 58.3%, compared to respectively 82.5% and 50.8% when using PCA3 cutoff score 25. The biomarker panel PCA3 (25) + TMPRSS2-ERG showed an increased sensitivity of 88.1% without compromising the specificity of PCA3 (49.6%). Sensitivity and specificity were not significantly different for the subgroup of patients without previous biopsies (data not shown).

Clinical implications

Based on serum PSA, all men in this study cohort underwent prostate biopsies. Table 6 shows the number of prostate biopsies that would be avoided and prostate cancer that would be missed if the urinary biomarker panel would be used to select men for prostate biopsies. The biomarker panel PCA3 + TMPRSS2-ERG was indicated positive if TMPRSS2-ERG and/or PCA3 was positive. The biomarker panel PCA3 (25) + TMPRSS2-ERG would avoid 35% of prostate biopsies, then 13% of prostate tumours would be missed. By combining PCA3 with TMPRSS2-ERG, an additional 13/443 men would be biopsied, of which 11 would be diagnosed with prostate cancer, including 9 men with Gleason score ≥ 7 .

Prostatectomy subcohort

In addition, we evaluated the subgroup of men (n=61) that underwent a radical prostatectomy (data not shown). Gleason score upon biopsy was upgraded in the prostatectomy specimen in 21% of men. TMPRSS2-ERG was an independent predictor of extracapsular extension (ECE) of the tumour upon radical prostatectomy (OR: 4.98, 95% CI: 1.13-21.98, p=0.034), serum PSA and PCA3 were not. None of the biomarkers correlated significantly with prostatectomy Gleason score or seminal vesicle invasion (n=8). The biomarker panel PCA3 + TMPRSS2-ERG did not correlate with any of the above-mentioned prognostic parameters upon prostatectomy.

Table 6 Clinical implications of the urinary biomarkers

	Prostate biopsies avoided (n=443) n (%)	Prostate cancers missed (n=196) n (%)	Prostate cancers Gleason ≥ 7 missed (n=115) n (%)
PCA3 score ≥ 25	166 (37%)	37 (19%)	20 (17%)
PCA3 score ≥ 35	211 (48%)	62 (32%)	36 (31%)
TMPRSS2-ERG ≥ 10	382 (86%)	150 (77%)	75 (65%)
PCA3-25 + TMPRSS2-ERG	153 (35%)	26 (13%)	11 (10%)
PCA3-35 + TMPRSS2-ERG	195 (44%)	48 (24%)	24 (21%)

PCA3 score = [copies PCA3 mRNA] / [copies PSA mRNA] x 1000

TMPRSS2-ERG positive = ≥ 10 copies TMPRSS2-ERG mRNA

PCA3-25 + TMPRSS2-ERG = TMPRSS2-ERG positive and/or PCA3 ≥ 25

PCA3-35 + TMPRSS2-ERG = TMPRSS2-ERG positive and/or PCA3 ≥ 35

DISCUSSION

In our study, PCA3 was a highly accurate biomarker for predicting prostate cancer, exceeding the performance of the widely used serum PSA. The optimal PCA3 cutoff score is still subject to debate. Several studies suggested lowering the cutoff score from 35 to 25⁵⁻⁸. In this study, the sensitivity for diagnosis clinically significant prostate cancer increased remarkably when lowering the cutoff score to 25 (68% vs. 83%). Concurrently, the specificity decreased from 58% to 51%. Defining the 'optimal' cutoff score will always be a compromise between sensitivity and specificity, depending on what 'risk' of missing prostate cancer is clinically acceptable. Based on the intended use, the optimal cutoff point can be determined based on the need for a high NPV or PPV in a particular setting (eg excluding cancer in a clinical cohort (high sensitivity/NPV), or a for population based screening (high specificity/PPV). Based on our results, a PCA3 cutoff score of 25 might be optimal; also when combined with TMPRSS2-ERG.

Furthermore, TMPRSS2-ERG was highly specific (93.2%) for predicting clinically significant prostate cancer upon biopsy. Therefore, when combining PCA3 with TMPRSS2-ERG, this will not compromise the specificity of PCA3. This makes TMPRSS2-ERG a valuable marker to combine in a panel with PCA3. Because of its specificity, reaching nearly 100%, in our opinion, TMPRSS2-ERG positive patients without prostate cancer upon biopsy would need immediate re-biopsy or MRI imaging. However, TMPRSS2-ERG is not yet validated as a prostate cancer biomarker to indicate for re-biopsy. Thus, we cannot use this test for this indication in daily practice (yet).

Both PCA3 and TMPRSS2-ERG had independent additional predictive value to the ERSPC risk calculator parameters for predicting prostate cancer. And if this biomarker panel PCA3 + TMPRSS2-ERG would have been used on this study cohort to select men for prostate biopsies, a substantial amount of (unnecessary) prostate biopsies (35%) would have been avoided, missing only 10% of the men with a Gleason score ≥ 7 prostate cancer. Our results confirm and validate the results of Hessels et al on this novel biomarker panel for prostate cancer¹⁴. Recently, Tomlins et al demonstrated that TMPRSS2-ERG in combination with PCA3 improved the performance of the multivariate PCPT risk calculator¹⁵. The limitation of their study was that it was not prospectively conducted and that for the centres not the same assays thresholds were used.

Knowing that Gleason score has high inter-pathologist variability, we let one experienced genitourinary pathologist review all biopsy Gleason scores independently. This resulted in a 4% downgrading of the Gleason score, and Gleason score was upgraded in 28% of

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reviewed biopsies. However, the pathology review did not change significance of our results (data not shown). When evaluating its potential prognostic value, PCA3 was not correlated with biopsy Gleason score and clinical tumour stage. When evaluating a subgroup of men that underwent a radical prostatectomy, again, PCA3 had no correlation with established risk factors. Our results are comparable to several large studies that have been published recently, showing that PCA3 had no additional predictive value for Gleason score and tumour stage^{5,8-10,19}. Although these studies showed PCA3 to be correlated with insignificant prostate cancer and tumour volume, the additional prognostic value of PCA3 is most likely limited.

This study demonstrates that the TMPRSS2-ERG urine assay had significant additional value to predict biopsy Gleason score, clinical tumour stage, and extracapsular extension of the tumour in the radical prostatectomy specimen. This may indicate that the TMPRSS2-ERG urine test could be used to select men with clinically significant prostate cancer. However, TMPRSS2-ERG was not correlated with Gleason score in radical prostatectomy specimens. Thus, the prognostic value of TMPRSS2-ERG in this subgroup of patients may be limited and needs further evaluation. The prognostic value of TMPRSS2-ERG when measured in tissue has been extensively studied, and the results are conflicting²⁰⁻²⁶. The hypothesis that TMPRSS2-ERG has prognostic value when measured in urine could be explained by the fact that aggressive prostate cancer cells are more invasive and invade surrounding structures, including the prostatic ductal system. Therefore, aggressive prostate cancers are considered to shed their cells more easily into the prostatic ductal system after DRE, resulting in a urine specimen with more TMPRSS2-ERG mRNA.

Our cohort consisted of a relatively small proportion of patients with clinically insignificant prostate cancer. This raises the question whether our study consists of a representative cohort. However, we have to emphasize that our study is a multicenter prospective trial and we analyzed an intent-to-use cohort, thus, per definition the selection bias is minimal. Our results might not be applicable to a screening cohort. Though, when analyzing the subcohort of patients with a normal DRE, sensitivity and specificity for PCA3, TMPRSS2-ERG and the panel, did not change significantly (data not shown). Furthermore, of the 126 patients with an abnormal DRE, 24% had no prostate cancer upon biopsies. This shows that DRE is very subjective, therefore, we don't think it is appropriate to preselect a population on basis of this criterium (DRE). The intention of the paper is to use objective criteria to diagnose patients. Biomarkers particularly fit this purpose.

CONCLUSIONS

In this prospective multicentre study we evaluated two novel urinary biomarkers for prostate cancer, ProgenSA PCA3 and TMPRSS2-ERG had independent additional predictive value to PCA3 and ERSPC risk calculator parameters for predicting prostate cancer. In addition, TMPRSS2-ERG had prognostic value. By implementing the novel biomarker panel PCA3 and TMPRSS2-ERG into clinical practice, this would lead to a considerable reduction of prostate biopsies.

Acknowledgements

The authors gratefully acknowledge Annelies Hoebers for performing ProgenSA PCA3 testing, Pim Peelen for collection of the samples, and Jan Hendriks for statistical assistance.

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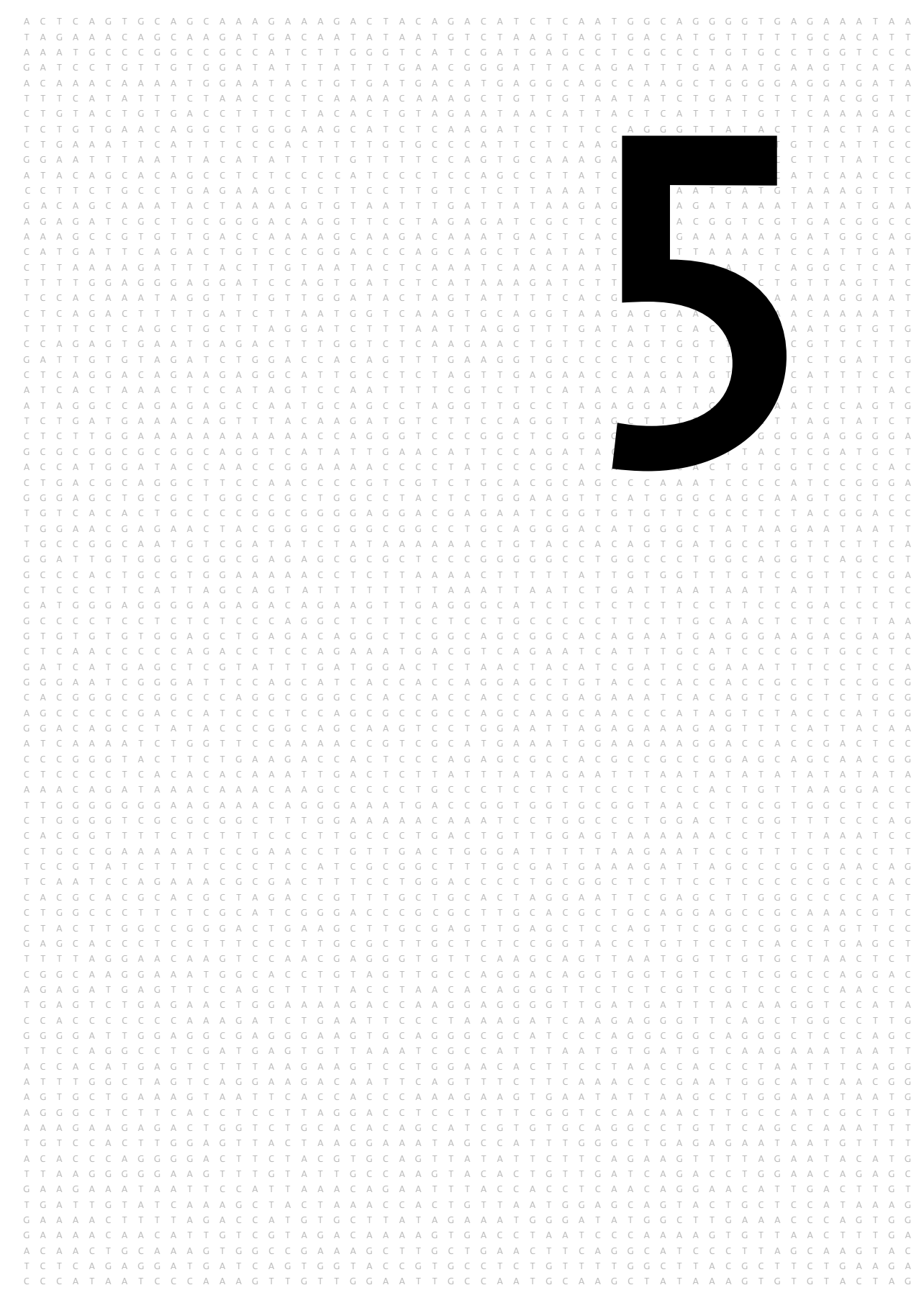
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QUATTRO, a four
gene prognostic biomarker
panel for prostate cancer

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Submitted

ABSTRACT

Introduction

The widespread use of serum PSA has led to the identification of patients with clinically indolent disease, inevitably over treatment has become a concern. The use of a urine test, ProgenSA PCA3, was shown to aid in the diagnosis of prostate cancer and prevents unnecessary prostate biopsies. However, its prognostic value seems limited. Therefore, biomarkers that can be used to identify patients with clinically significant disease (Gleason score ≥ 7) are urgently needed.

Material and Methods

Using microarray analysis on snap frozen tissue, 46 candidate biomarkers were selected. With Taqman low density arrays (TLDA), we identified ten most promising biomarkers (*HOXC4*, *HOXC6*, *DLX1*, *TDRD1*, *ONECUT2*, *NKAIN1*, *MS4A8B*, *PTPRT*, *RRM2* and *ACSM1*). This lead to the hypothesis that a combination of these ten biomarkers is superior to sPSA and PCA3 to identify patients with Gleason score ≥ 7 prostate cancer. This hypothesis was tested in a prospective multicenter study on urine specimens of 358 men undergoing prostate biopsies.

Results

A four gene panel was selected in the clinical study: *HOXC4*, *HOXC6*, *TDRD1* and *DLX1*, referred to as QUATTRO. QUATTRO had a predictive accuracy for the diagnosis of Gleason score ≥ 7 of 0.78; which was evidently higher than for sPSA (0.72) and ProgenSA PCA3 (0.69). When sPSA was added to QUATTRO, the predictive accuracy increased to 0.82.

Conclusions

We describe a stepwise development of a four gene-based test (QUATTRO), that was superior to sPSA and PCA3 to identify patients with Gleason score ≥ 7 prostate cancer.

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INTRODUCTION

With the introduction of serum prostate-specific antigen (PSA) testing in the late 1980s, the incidence of prostate cancer has increased considerably¹. Worldwide, 899,102 men are diagnosed with prostate cancer every year of whom 258,133 men die from this disease². However, the serum PSA (sPSA) test has a low specificity for prostate cancer, as conditions such as benign prostatic hyperplasia (BPH) and prostatitis can also lead to an elevated sPSA level. This results in high negative biopsy rates of 70-80% in the so-called ‘grey area’ of PSA levels 4.0-10.0 ng/ml^{3,4}. Moreover, PSA-based (opportunistic) screening has led to the diagnosis of clinically insignificant prostate tumours, *i.e.* in the absence of screening, these tumours would not have been diagnosed within the patient’s lifetime, which results in over-treatment⁵. Currently, it is difficult to predict which tumour will become potentially life threatening and which one will not. Therefore, overtreatment of localized prostate cancer is a serious clinical issue causing morbidity and unnecessary health care costs. Prognostic biomarkers with a much higher specificity than PSA are urgently needed to improve the detection of clinically significant prostate cancer. Clinically significant prostate cancer is defined as: Gleason score ≥ 7 , non-organ confined disease ($>T_2$ disease) and tumour volume $>0.5\text{ cm}^3$ ⁶. However, as digital rectal examination (DRE) and transrectal ultrasound (TRUS) findings are only moderately reproducible and not reliable for predicting tumour stage and tumour volume, these two parameters cannot be reliably determined on biopsied patients⁷. Therefore, Gleason score ≥ 7 remains the most powerful parameter for clinically significant prostate cancer for biopsied patients.

In this search for prostate cancer specific biomarkers, two promising candidates have been identified: Prostate CAncer gene 3 (*PCA3*) and *TMPRSS2-ERG* gene fusions. These biomarkers can be measured using a non-invasive urine test. The *PCA3* gene is highly over-expressed in prostate tumours⁸, and has diagnostic value to predict biopsy outcome⁹⁻¹¹, but its prognostic value is limited¹²⁻¹⁴. The Progenesa® *PCA3* test is an FDA-approved molecular diagnostic test that is available to urologists. Gene fusions in which ETS family members are mostly fused to androgen-regulated genes, particularly *TMPRSS2*, are prostate cancer-specific molecular events. *TMPRSS2-ERG* gene fusions are present in approximately 50% of prostate cancer patients^{15,16}. The prognostic value of this gene fusion is still unclear¹⁷⁻²⁰. Consequently, the urgent need for more accurate *prognostic* biomarkers for prostate cancer persists. To address this unmet need, the stepwise development of a four gene based test is described in this manuscript. Using microarray analysis on snap frozen tissue, 46 candidate biomarkers were selected.

With Taqman low density arrays (TLDA), we identified ten most promising candidate biomarkers. This led to the hypothesis that a combination of these ten biomarkers is superior to PCA3 to identify patients with Gleason score ≥ 7 prostate cancer. This hypothesis was tested in an independent cohort in our prospective multicenter study on urine specimens of men undergoing prostate biopsies.

MATERIAL AND METHODS

Preclinical biomarker discovery

Microarray analysis

Prostate tissue specimens were collected at the Radboud University Nijmegen Medical Centre and Canisius Wilhelmina Hospital Nijmegen. This study was approved by the local ethics committees of both centres.

In total, tissue specimens of 133 patients were collected for both microarray as well as TLDA. We obtained samples with normal prostate (NP; $n=12$), BPH ($n=16$), low grade prostate cancer (LG-PCa; $n=33$), high grade prostate cancer (HG-PCa; $n=32$), castration resistant prostate cancer (CRPC; $n=32$) and metastatic prostate cancer (PCa-M+; $n=8$). Samples were obtained after transurethral resection of the prostate (TURP), open adenectomy, radical prostatectomy and lymph node dissection. After collection, all specimens were snap frozen in liquid nitrogen and cryostat sections were hematoxylin and eosin (HE) stained for classification by a pathologist.

Ninety nine samples were used for whole transcriptome analysis (GeneChip® human exon 1.0 ST array (Affymetrix)); after bioinformatic analyses and data interpretation. Forty six candidate genes were selected for TLDA analysis on a case mix of 73 microarray and 34 new prostate tissue specimens.

RNA isolation

Tumour- and tumour-free areas were dissected and total RNA was extracted by using TRIzol reagent (Invitrogen) according to manufacturer's instructions. Total RNA was DNase treated and purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The integrity of the RNA was determined using the Agilent 2100 Bioanalyzer. Samples with RNA integrity number (RIN) ≥ 6 were included for microarray analysis.

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Expression analysis

Gene expression profiles were determined using the GeneChip® Human Exon 1.0 Sense Target (ST) arrays (Affymetrix) according to the manufacturer's protocol. One µg of RNeasy purified total RNA was used to generate amplified and biotinylated sense-strand DNA targets from the entire expressed genome. According to the protocol, the majority of ribosomal RNA was removed using the RiboMinus Human/Mouse Transcriptome Isolation Kit (Invitrogen). The generated amplified sense-strand cDNA targets were fragmented by incubation with a mixture of UDG (uracil DNA glycosylase) and APE1 (apurinic/apyrimidinic endonuclease 1) restriction endonucleases and end-labelled via a terminal transferase reaction incorporating a biotinylated dideoxynucleotide. Of the fragmented, biotinylated cDNA, 5.5 µg was added to a hybridization mixture, loaded on a GeneChip® Human Exon 1.0 ST (Affymetrix) and hybridized for 16 hours at 45°C and 60 rpm. Following hybridization, the array was washed in a GeneChip® Fluidics station FS 450 (Affymetrix) and stained according to the Affymetrix protocol. The array was scanned at 532 nm using an GeneChip Scanner 3000 7G (Affymetrix), generating CEL files for each array.

Gene-level and exon-level expression values were derived from the CEL file using the model-based Robust Multiarray Average (RMA) algorithm as implemented in Partek software (Partek Genomics Suite 6.6). RMA performs normalization, background correction and data summarization. P-values of differentially expressed genes between conditions was calculated using ANOVA analysis. For the identification of biomarkers the expression analysis of the different groups were compared: NP/BPH with LG- and HG-PCa, PCa-M+ with LG- and HG-PCa, CRPC with LG- and HG-PCa.

Taqman® Low Density Arrays

Further selection of the biomarkers was performed using Applied Biosystems Taqman® Low Density Arrays (TLDA). A format to screen 46 different genes per sample was chosen to analyze a total of 107 prostate tissue samples. Data of GeneChip Microarray and TLDA analysis are shown in Table 1. 2 µg of RNA was used in cDNA synthesis using SuperScript III (Invitrogen) according to the manufacturer's instructions. One twentieth of the cDNA synthesis was mixed with Taqman® Universal PCR Master Mix (Applied Biosystems) and was loaded on the TLDA card. The card was run on a Applied Biosystems 7900 HT thermal cycler with default thermal cycling conditions for a 384 wells TLDA card as set in the SDS software. TLDA $\Delta\Delta C_t$ analysis was performed using RQ study software (Applied Biosystems). Based on these results ten most promising biomarkers were selected for quantitative PCR analysis on urine specimen.

Table 1 GeneChip Microarray and TLDA data

Gene symbol	Gene name	GeneChip Microarray data		TLDA data
		Fold-Change NP vs LG+HG	P-value* NP vs LG+HG	Fold-Change NP vs LG+HG
ONECUT2	one cut homeobox 2	14.3	8.4 E-07	n.a.
RRM2	ribonucleotide reductase M2 polypeptide	8.6	7.9 E-07	8.1
ACSM1	acyl-CoA synthetase medium-chain family member 1	8.4	4.8 E-04	7.4
TDRD1	tudor domain containing 1	7.7	2.9 E-03	139.0
MS4A8B	membrane-spanning 4-domains,subfam. A, member 8B	5.0	1.0 E-05	16.6
HOXC6	homeobox C6	4.6	5.0 E-04	10.5
PTPRT	protein tyrosine phosphatase, receptor type, T	4.2	1.1 E-03	21.2
NKAIN1	Na ⁺ /K ⁺ transporting ATPase interacting 1	3.8	2.9 E-03	17.6
DLX1	distal-less homeobox 1	3.5	1.6 E-05	39.0
HOXC4	homeobox C4	2.3	2.5 E-03	n.a.
CRISP3	cysteine-rich secretory protein 3	17.1	3.9 E-03	68.5
GLYATL1	glycine-N-acyltransferase-like 1	10.2	1.7 E-08	12.6
AMACR	alpha-methylacyl-CoA racemase	9.6	5.7 E-08	58.0
TMEM45B	transmembrane protein 45B	7.1	3.4 E-05	13.9
FOLH1	folate hydrolase (prostate-spec. membrane antigen) 1	6.5	7.3 E-04	5.4
C19orf48	chromosome 19 open reading frame 48	5.9	4.1 E-09	3.7
ALDH3B2	aldehyde dehydrogenase 3 family, member B2	5.7	4.4 E-06	24.0
NETO2	neuropilin (NRP) and tolloid (TLL)-like 2	5.6	2.0 E-06	11.3
TLCD1	TLC domain containing 1	4.7	1.3 E-10	4.0
FASN	fatty acid synthase	4.7	1.2 E-07	3.9
GRPR	gastrin-releasing peptide receptor	4.5	1.4 E-03	5.2
TOP2A	topoisomerase (DNA) II alpha 170kDa	3.8	1.2 E-04	8.7
FAM111B	family with sequence similarity 111, member B	3.8	2.7 E-04	2.9
TPX2	TPX2, microtubule-associated, homolog (Xenopus laevis)	3.5	5.5 E-05	12.2

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Gene symbol	Gene name	GeneChip Micro array data		TLDA data
		Fold-Change NP vs LG+HG	P-value* NP vs LG+HG	Fold-Change NP vs LG+HG
CGREF1	cell growth regulator with EF-hand domain 1	3.2	4.9 E-07	7.0
PPFIA2	protein tyrosine phosphatase, receptor type, f polypept	3.0	3.2 E-02	8.5
ERG	v-ets erythroblastosis virus E26 oncogene homolog	3.0	8.7 E-02	26.7
MKI67	antigen identified by monoclonal antibody Ki-67	3.0	5.2 E-04	7.9
COMP	cartilage oligomeric matrix protein	3.0	1.7 E-03	15.1
PTTG1	pituitary tumour-transforming 1	2.7	1.3 E-03	4.1
CKS2	CDC28 protein kinase regulatory subunit 2	2.6	2.1 E-03	3.0
ANLN	anillin, actin binding protein	2.2	3.7 E-03	4.6
KIF20A	kinesin family member 20A	1.9	1.1 E-02	15.5
KIF4A	kinesin family member 4A	1.8	1.8 E-02	14.8
BUB1	budding uninhibited by benzimidazoles 1 homolog	1.8	7.1 E-03	4.6
CDC20	cell division cycle 20 homolog (S. cerevisiae)	1.7	1.2 E-01	9.3
FAM110B	family with sequence similarity 110, member B	1.7	2.9 E-01	2.0
TTN	titin	1.5	4.6 E-01	0.7
UGT2B15	UDP glucuronosyltransferase 2 family, polypeptide B15	1.3	5.8 E-01	14.0
NR4A1	nuclear receptor subfamily 4, group A, member 1	1.1	7.7 E-01	2.0
HSPB8	heat shock 22kDa protein 8	-2.4	2.0 E-04	0.5
PGM5	phosphoglucomutase 5	-4.4	3.7 E-07	0.5
ASPA	aspartoacylase (Canavan disease)	-5.2	4.6 E-12	0.3
MT1M	metallothionein 1M	-5.8	6.8 E-04	0.7
DPT	dermatopontin	-6.3	6.4 E-09	0.4
HPRT1	hypoxanthine phosphoribosyltransferase 1	1.1	9.9 E-02	1.0

NP=normal prostate tissue, LG = Gleason score ≤6 prostate cancer, HG = Gleason score ≥7 prostate cancer.*
To test for statistical significance, one-way ANOVA analysis was used

Clinical prospective study

Study design

We designed a prospective, multicenter study, to test the hypothesis that (a combination of) candidate biomarkers that were selected in the pre-clinical biomarker discovery study is superior to the established biomarkers sPSA and PCA3 to identify patients with Gleason score ≥ 7 prostate cancer upon biopsy. Men who were scheduled for (initial or repeat) prostate biopsies, based on elevated sPSA levels, a family history of prostate cancer or an abnormal DRE were included. First-catch urine after DRE was collected from 443 men, as described by Groskopf et al⁹. Prostate biopsies were performed and evaluated per hospital's standard procedure (9-12 biopsy cores). In addition, one experienced genitourinary pathologist (CAH-K) reviewed all biopsy Gleason scores independently, being blinded for the biomarker scores. Men were recruited at six urology clinics in the Netherlands (Radboud University Nijmegen Medical Centre, Nijmegen; Academic Medical Centre, Amsterdam; ZGT Hospital, Hengelo; Canisius Wilhelmina Hospital, Nijmegen; Scheper Hospital, Emmen; and St. Elisabeth Hospital, Tilburg). Exclusion criteria were: history of prostate cancer, medical therapy known to affect sPSA levels, prostate biopsies within three months prior to enrolment, or invasive treatment for BPH within six months prior to enrolment. The respective independent ethics committees approved the study protocol and all included patients provided written informed consent. The biomarker discovery and the clinical validation study were both performed in accordance with the STARD (*ST*Andards for Reporting of Diagnostic accuracy) criteria and REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) guidelines^{21,22}.

Data collection

Clinicopathological data were collected for each patient, including: age, sPSA, DRE and TRUS results, prostate volume, biopsy results (current and history), radiological results, clinical TNM stage (if diagnosed with prostate cancer) and radical prostatectomy results (if applicable). These data and the assay results were entered in a secured preset web-based database with audit trail (in compliance with the International Conference on Harmonization-Good Clinical Practice guidelines). Assay results were not provided to the clinical sites for patient care and the technicians that performed the assays were blinded for patient characteristics.

Specimen processing

First-catch urine specimens after DRE were processed using a validated standard operating procedure (SOP). This SOP is an integration of procedures for whole urine, described by Groskopf et al⁹, and urinary sediments as described by Hessels et al²³.

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In short, immediately, 2.5 ml of the first voided urine after DRE was transferred to a ProgenSA® PCA3 urine sample collection tube containing sample transport medium (Gen-Probe Inc.). And urine was collected in a coded container with 4 ml 0.5 M EDTA. The urine samples were immediately cooled to 4°C and were mailed with cold packs to NovioGendix Research BV (Nijmegen, The Netherlands). Urine samples were processed within 48 h after collection to guarantee good sample quality. The urine, EDTA stabilized, was centrifuged at 4°C and 1.800 x g for 10 minutes. The obtained urinary sediments were washed twice with ice-cold buffered sodium-chloride solution, and snap frozen in liquid nitrogen and stored at -70°C.

Quantitative real-time PCR

RNA was extracted from the urinary sediments using a modified Tripure reagent (Roche, Cat no. 11.667.165.001) protocol. After the chloroform extraction, GlycoBlue (Ambion, Cat no. AM 9515) was added to the aqueous phase to precipitate the RNA using isopropanol (Merck, Cat No. 1.00994.1001). The RNA samples that were obtained were DNase treated prior to the amplification protocol using DNase I enzyme (Invitrogen, Cat. No.18068-015). The RNA samples were purified using ethanol (Merck, Cat No 1.00983)/sodium acetate precipitation (Ambion, Cat no. AM 9740). Total RNA from the sediments was used to generate amplified sense-strand cDNA using the Whole Transcriptome (WT) Expression Kit (Ambion, Cat no. 4411974) according to the manufacturers protocol. Fluorescence based real-time PCR assays were designed by and manufactured by TIB Molbiol Berlin. PCR products were cloned in either the pCR®-Blunt cloning vector (Invitrogen) or the pCR2.1-TOPO cloning vector (Invitrogen). Calibration curves with a wide linear dynamic range (10 – 1.000.000 copies) were generated using serial dilutions of the plasmids. The amplification efficiency of the primer pair was determined using the calibration curve and was >1.85. Control samples with known template concentrations were used as a reference. Two µl of each cDNA sample were amplified in a 20 µl PCR reaction containing optimized amounts of forward primer and reverse primer, 2 pmol of hydrolysis probe and 1x Probes Master mix (Roche, Cat No. 04902343001). The following amplification conditions were used: 95°C for 10 minutes followed by 50 cycles at 95°C for 10 seconds, 60°C for 30 seconds and a final cooling step at 40°C for 55 seconds (LightCycler LC480, Roche). The crossing point (Cp) values were determined using the Lightcycler 480 SW 1.5 software (Roche). The Cp values of the samples were converted to concentrations by interpolation in the generated calibration curve. The assay performance of the real-time PCR experiments was evaluated during in-study validation. The reference control samples had an inter- and intra-assay variation < 30%.

KLK3, mPCA3, TMPRSS2-ERG, HPRT and the ten most promising biomarkers that were

identified in the biomarker discovery study were measured. Urine specimens were classified as passing quality control if the copy number of HPRT for the urinary sediment was ≥ 4000 copies. If this threshold was not met, the specimen was classified as failing quality control and excluded. The urine in the Progenisa® PCA3 urine sample collection tube was used for the Progenisa® PCA3 test at NovioGendix as described by Groskopf et al⁹ The PCA3 score was calculated as $[\text{PCA3 mRNA}]/[\text{PSA mRNA}] \times 1000$.

Statistical analyses

Statistical analyses were performed with SPSS® version 20.0. Two-sided P values of ≤ 0.05 were considered to indicate statistical significance. Fisher's exact test (for dichotomous variables) and the nonparametric Mann Whitney test (for continuous variables) were used to test if biomarker levels were significantly correlated with prostate cancer, Gleason score and clinical tumour stage. PCA3 score was assessed both as a continuous and as a dichotomous biomarker with cut-off of 25 and 35. *TMPRSS2-ERG* was used as a dichotomous biomarker, all samples with ≥ 10 copies *TMPRSS2-ERG* mRNA were marked as positive, as this is the lower limit of detection of the assay. sPSA and the novel biomarkers were assessed as continuous biomarkers. Forward logistic regression analysis was used to test if the novel biomarkers had independently additional predictive value ($p < 0.05$) to sPSA and PCA3 for diagnosis prostate cancer and Gleason score ≥ 7 prostate cancer diagnosis. The Odds Ratios and AUCs and the corresponding 95% Confidence Intervals (CI) of the final model are presented.

The statistical design was based on the assumption of an $\text{AUC} = 0.73$ for the novel biomarker panel, compared to an $\text{AUC} = 0.65$ for PCA3. With a type 1 error (α) of 0.05 and a power to detect this difference of 80%, we needed a sample size of 350 evaluable urinary samples.

RESULTS

Biomarker discovery on tissue specimen

To identify candidate biomarkers, we used the Affymetrix exon array platform to molecularly profile prostate cancer tissue specimens in several categories (NP, BPH, LG PCa, HG PCa, CRPC and M+ PCa). Interactive bioinformatic analysis combining outlier analysis, fold change and p-value led to the identification of 46 candidate biomarkers. These were analysed in a new case mix using TLDA. Figure 1 schematically illustrates the stepwise approach of this study. In this way we identified ten most promising candidate biomarkers (*HOXC4*, *HOXC6*, *DLX1*, *TDRD1*, *ONECUT2*, *NKAIN1*, *MS4A8B*, *PTPRT*, *RRM2* and

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ACSM¹). With these markers, we tested the hypothesis that (a combination of) these ten candidate biomarkers is superior to the established biomarkers sPSA and PCA3 to identify patients with Gleason score ≥ 7 prostate cancer upon biopsy in urine specimen.

Clinical prospective study on urine after DRE

The hypothesis was tested in a prospective study on first catch urine specimen after DRE. In total, 443 patients were enrolled in this prospective multicenter study between September 2009 and July 2011. Samples that had *HPRT* mRNA < 4000 copies were excluded for this study (n=85). This resulted in 358 evaluable samples. Patient characteristics are shown in Table 2. Prostate cancer was diagnosed in 44% of patients, of which 59% had prostate cancer with a Gleason score ≥ 7 .

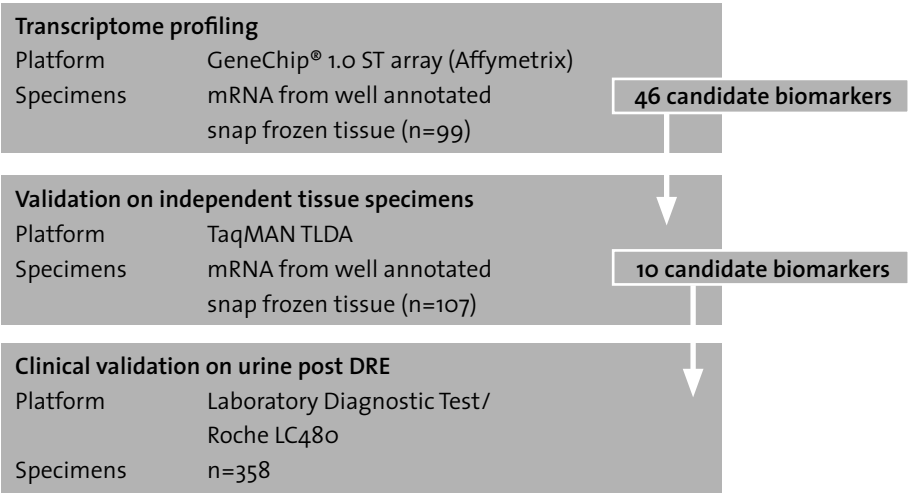


Figure 1 Study design. A stepwise approach for the identification and selection of a prognostic gene panel. The pre-clinical biomarker discovery study in snap frozen tissue. Then, the hypothesis that a combination of the ten selected most promising biomarkers is superior to the established biomarkers sPSA and PCA3 was tested in the clinical prospective study on urine specimen of 358 men undergoing prostate biopsies

Table 2 Patient characteristics of clinical prospective study, evaluation of urine specimens of 358 patients

Descriptives	Cohort (n= 358) median (range) / n (%)
Age	65 (44-86)
Prostate cancer in family	66 (18%)
No previous biopsies	280 (78%)
Abnormal DRE	103 (61%)
TRUS prostate volume (cc)	48 (15-200)
Prostate cancer upon biopsy	157 (44%)
Gleason score ≥ 7	93 (26%)

DRE = Digital Rectal Examination

TRUS = TransRectal UltraSound

Table 3 shows the biomarker characteristics per biopsy outcome: prostate cancer, significant prostate cancer and Gleason score ≥ 7 prostate cancer. All biomarkers, except *RRM2*, were expressed significantly higher in patients with prostate cancer compared to patients without prostate cancer. In patients with Gleason score ≥ 7 prostate cancer diagnosis upon biopsy, all biomarker levels were significantly higher compared to the rest of patients (Gleason score ≤ 6 prostate cancer or biopsy negative patients). *HPRT* was not significantly correlated with each outcome (negative control).

sPSA, and mRNA levels of *HOXC4*, *HOXC6*, *DLX1*, *TDRD1*, *ONECUT2*, *NKAIN1*, *MS4A8B* and *PTPRT* were significantly higher in patients with Gleason score ≥ 7 compared to those with Gleason score ≤ 6 prostate cancer, whereas Progenisa PCA3 score was not. When evaluating the subgroup of patients with significant prostate cancer: Progenisa PCA3 score and *TMPRSS2-ERG* expression levels were not discriminative between patients with significant and insignificant prostate cancer. sPSA, *HOXC6*, *DLX1*, *TDRD1*, *ONECUT2* and *PTPRT* were significantly higher in patients with significant prostate cancer compared to patients with insignificant prostate cancer.

Forward logistic regression was used to identify the best combination of markers. sPSA and the ten novel markers were log transformed, only *HOXC6* (OR 1.40; 95% CI 1.14-1.72; $p=0.001$), *TDRD1* (OR 1.13; 95% CI 1.01-1.28; $p=0.038$), and *DLX1* (OR 1.15; 95% CI 1.01-1.30;

Table 3a Biomarker characteristics by prostate cancer, significant prostate cancer and Gleason score

	Prostate cancer			Significant Prostate cancer*		
	No (n=201) median (range) / n (%)	Yes (n=157) median (range) / n (%)	p-value	Insignificant PCa (n=19) median (range) / n (%)	Significant PCa (n=138) median (range) / n (%)	p-value
Age	64 (46-80)	65 (44-86)	0.022 ^a	65 (52-79)	65 (44-86)	0.583 ^a
Serum PSA (ng/ml)	6.8 (0.4-48)	9.2 (0.6-756)	<0.001 ^a	5.5 (0.6-12)	9.6 (2.3-756)	<0.001 ^a
PCA3 score	24 (0-219)	60 (0-522)	<0.001 ^a	54 (15-308)	60 (0-522)	0.893 ^a
PCA3 score ≥5	97 (48.3%)	125 (79.6%)	<0.001 ^b	14 (73.7%)	111 (80.4%)	0.544 ^b
PCA3 score ≥35	84 (41.8%)	105 (66.9%)	<0.001 ^b	14 (73.7%)	91 (65.9%)	0.609 ^b
mPCA3	1170 (1-172000)	5280 (1-359000)	<0.001 ^a	7150 (316-27200)	5085 (1-359000)	0.653 ^a
TMPS52-ERG ≥10	12 (6.0%)	40 (25.5%)	<0.001 ^b	3 (15.8%)	37 (26.8%)	0.405 ^b
KLK3	93700 (1320-7700000)	182000 (2360-48350000)	<0.001 ^a	188000 (34200-1515000)	177000 (2360-48350000)	0.955 ^a
HPRT	29300 (4010-738000)	31800 (4070-529000)	0.871 ^a	32900 (4440-522000)	31650 (4070-529000)	0.788 ^a
HOXC4	5260 (1-116000)	12600 (1-355000)	<0.001 ^a	7870 (563-46700)	13850 (1-355000)	0.213 ^a
HOXC6	321 (1-8970)	962 (1-193000)	<0.001 ^a	443 (29-5250)	1195 (1-193000)	0.010 ^a
DLX1	1 (1-3690)	1 (1-66700)	<0.001 ^a	1 (1-161)	1 (1-66700)	0.040 ^a
TDRD1	124 (1-56400)	367 (1-727000)	<0.001 ^a	34 (1-1020)	447 (1-727000)	<0.001 ^a
RRM2	7740 (235-327000)	9760 (309-172000)	0.193 ^a	9820 (365-88900)	9745 (309-172000)	0.520 ^a
ONECUT2	776 (1-8100)	1280 (1-207000)	<0.001 ^a	464 (12-4740)	1570 (1-207000)	0.001 ^a
NKAIN1	162 (1-3620)	291 (1-84800)	<0.001 ^a	190 (1-1630)	319.5 (1-84800)	0.198 ^a
MS4A8B	168 (1-203000)	612 (1-401000)	<0.001 ^a	475 (1-2100)	679.5 (1-401000)	0.086 ^a
PTPRT	1 (1-5420)	10 (1-265000)	<0.001 ^a	1 (1-286)	10 (1-265000)	0.003 ^a
ACSM1	297 (1-4800)	594 (1-264000)	0.001 ^a	756 (39-2180)	591 (1-264000)	0.276 ^a

* Clinically significant prostate cancer according to Epstein criteria: clinical stage ≥T2, Gleason score ≥7, PSAD >0.15 and >33% positive cores.
^a = Mann Whitney test. ^b = Fishers exact test.

Table 3b Biomarker characteristics by prostate cancer, significant prostate cancer and Gleason score

	Gleason score			Gleason score			
	≤6 (n=64) median (range) / n (%)	≥7 (n=93) median (range) / n (%)	p-value	REST (n=265) median (range) / n (%)	≥7 (n=93) median (range) / n (%)	p-value	AUC (95% CI)
Age	63 (52-85)	67 (44-86)	0.007 ^a	64 (46-85)	67 (44-86)	<0.001 ^a	0.626 (0.559-0.692)
Serum PSA (ng/ml)	8 (0.6-38)	10.8 (2.3-756)	<0.001 ^a	6.9 (0.4-48)	10.8 (2.3-756)	<0.001 ^a	0.715 (0.652-0.778)
PCA3 score	55.5 (2-308)	61 (0-522)	0.278 ^a	31 (0-308)	61 (0-522)	<0.001 ^a	0.683 (0.620-0.746)
PCA3 score ≥25	49 (76.6%)	76 (81.7%)	0.546 ^b	146 (55.1%)	76 (81.7%)	<0.001 ^b	0.632 (0.569-0.695)
PCA3 score ≥ 35	41 (64.1%)	64 (68.8%)	0.606 ^b	125 (47.2%)	64 (68.8%)	<0.001 ^b	0.607 (0.541-0.672)
mPCA3	4960 (1-144000)	5290 (1-359000)	0.304 ^a	1750 (1-172000)	5290 (1-359000)	<0.001 ^a	0.686 (0.624-0.748)
TPMRSS2-ERG ≥10	6 (9.4%)	34 (36.6%)	<0.001 ^b	18 (6.8%)	34 (36.6%)	<0.001 ^b	0.651 (0.580-0.722)
KLK3	166500 (2360-1590000)	227000 (4020-48350000)	0.073 ^a	104000 (1320-7700000)	227000 (4020-48350000)	<0.001 ^a	0.642 (0.572-0.713)
HPRT	28450 (4110-522000)	34000 (4070-529000)	0.173 ^a	29300 (4010-738000)	34000 (4070-529000)	0.287 ^a	0.536 (0.468-0.604)
HOXC4	8120 (134-59100)	14700 (1-355000)	0.034 ^a	5940 (1-116000)	14700 (1-355000)	<0.001 ^a	0.686 (0.619-0.753)
HOXC6	633 (16-11900)	1550 (1-193000)	<0.001 ^a	392 (1-11900)	1550 (1-193000)	<0.001 ^a	0.756 (0.697-0.815)
DLX1	1 (1-1900)	35 (1-66700)	<0.001 ^a	1 (1-3690)	35 (1-66700)	<0.001 ^a	0.702 (0.632-0.771)
TDRD1	159 (1-6970)	843 (1-727000)	<0.001 ^a	130 (1-56400)	843 (1-727000)	<0.001 ^a	0.734 (0.669-0.799)
RRM2	8280 (365-97600)	10800 (309-172000)	0.058 ^a	7740 (235-327000)	10800 (309-172000)	0.024 ^a	0.575 (0.507-0.643)
ONECUT2	1020 (1-8920)	1790 (1-207000)	<0.001 ^a	804 (1-8920)	1790 (1-207000)	<0.001 ^a	0.689 (0.625-0.752)
NKAIN1	192.5 (1-3050)	392 (1-84800)	0.006 ^a	163 (1-3620)	392 (1-84800)	<0.001 ^a	0.659 (0.588-0.730)
MS4A8B	471.5 (1-6020)	1010 (1-401000)	0.001 ^a	204 (1-203000)	1010 (1-401000)	<0.001 ^a	0.698 (0.632-0.764)
PTPRT	1 (1-531)	88.5 (1-265000)	<0.001 ^a	1 (1-5420)	88.5 (1-265000)	<0.001 ^a	0.696 (0.628-0.765)
ACSM1	478 (1-7790)	683.5 (1-264000)	0.308 ^a	338 (1-7790)	683.5 (1-264000)	0.007 ^a	0.595 (0.521-0.669)

REST = no prostate cancer + Gleason score ≤6. AUC = Area Under the Curve. 95% CI = 95% Confidence Interval.

^a = Mann Whitney test. ^b = Fishers exact test

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p=0.030), had independent additional predictive value to sPSA (OR 2.84; 95% CI 1.78-4.52; p<0.001), for predicting Gleason score ≥ 7 prostate cancer. The performance of a logistic model was judged on its goodness of fit and on its discriminatory power (AUC). Goodness of fit was calculated with the statistic H of Hosmer and Lemeshow, this showed that the model was well calibrated (p=0.490). Thus, of the ten novel biomarkers, *HOXC6*, *TDRD1* and *DLX1* were selected for further analysis.

In addition to the three novel markers *HOXC6*, *TDRD1* and *DLX1*, which are all independent predictors of patients with a Gleason score ≥ 7 prostate cancer diagnosis upon biopsy, *HOXC4* was added to the biomarker panel. *HOXC4* is expressed from the same transcription unit as *HOXC6*. *HOXC4* was also a significant diagnostic and prognostic biomarker, but in multivariate analysis it was not an independent predictor. This can be explained by the fact that it is transcribed from the same transcription unit as *HOXC6*. Therefore, the decision was made to build in some redundancy in the biomarker panel. This panel of *HOXC4*, *HOXC6*, *TDRD1* and *DLX1* is referred to as QUATTRO.

Using ROC curve analysis, QUATTRO had a predictive accuracy for the diagnosis Gleason score ≥ 7 prostate cancer upon biopsy of 0.78 (95% CI 0.72-0.84), which was evidently higher than for sPSA (AUC 0.72; 95% CI 0.66-0.78) and Progens PCA3 (AUC=0.69; 95% CI 0.63-0.75) (Figure 2). When QUATTRO was added to sPSA, the predictive accuracy increased to 0.82 (95% CI 0.76-0.87) vs. 0.79 (95% CI 0.74-0.84) when PCA3 was added to sPSA.

When entered in logistic regression analysis, the combination of sPSA and QUATTRO yielded the following model for the probability of a Gleason score ≥ 7 prostate cancer diagnosis upon biopsy in urine sediments:

$$Probability = 1 / (1 - EXP (-6.0787 + 1.039(\ln sPSA) - 0.075(\ln HOXC4) + 0.387(\ln HOXC6) + 0.139(\ln DLX1) + 0.123(\ln TDRD1)))$$

Where the units of measurements of the QUATTRO markers are copy numbers and sPSA is ng/ml. A probability of 0.297, with the use of this equation, was the cutoff point that maximized the combined sensitivity (66.3%) and specificity (84.5%)(Youlden's index) of the model.

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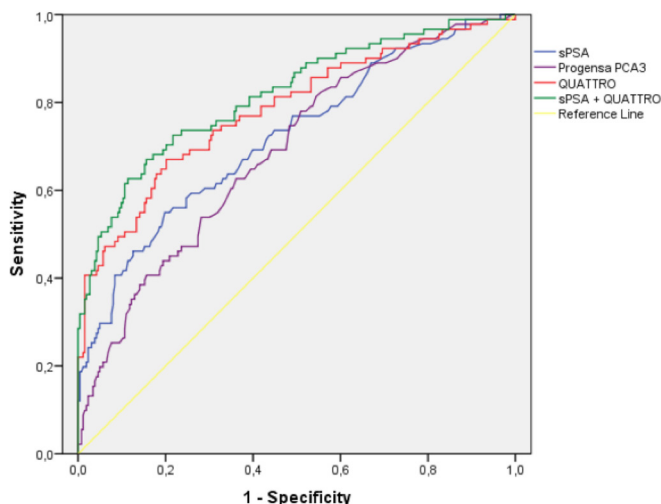


Figure 2 The ROC curves for four models: Progenia PCA3 (purple line, AUC = 0.69; 95% CI 0.63 – 0.75), sPSA (blue line, AUC = 0.72; 95% CI 0.66–0.78), QUATTRO (red line, AUC = 0.78; 95% CI 0.72 – 0.84) and sPSA + QUATTRO (green line, AUC = 0.82; 95% CI 0.76 – 0.87) for the prediction of Gleason score ≥ 7 prostate cancer diagnosis upon biopsy.

DISCUSSION

Nowadays, biomarker research gained considerable attention. Yet, only very few results appear reproducible. This can be partly explained by the fact that biomarker studies are not regulated by clear guidelines, and many studies do not meet the STARD criteria and are not reported in compliance with the REMARK guidelines^{21,22}. Our study does meet the STARD criteria and REMARK guidelines. This is a multicenter prospective study in an intended to use cohort, therefore, this cohort can be considered representative and a possible bias to be minimal.

Given the heterogeneous character of prostate cancer, a panel of biomarkers can be superior to the use of a single biomarker. A panel will supply a higher degree of redundancy. The combined use of Progenia PCA3 and TMPRSS2-ERG has previously been shown to increase diagnostic value of PCA3 alone²⁴. However, the limited prognostic value of Progenia PCA3 and the restricted number of prostate cancer patients with TMPRSS2-ERG gene fusions, makes this panel unsuitable for predicting Gleason score ≥ 7 prostate cancer diagnosis upon biopsy.

This study used a pragmatic approach to identify candidate biomarkers for an important clinical unmet need, *i.e.* a panel that can be used to identify patients with clinically significant prostate cancer. Our stepwise development of the four gene-based test can be considered a result of ‘rational design’ (Figure 1), rather than a pure biostatistical interpretation of expression data. Forty six candidate biomarkers were identified based on molecular profiling of prostate cancer tissue specimens. These candidate biomarkers were analysed in tissue specimen by TLDA, after which ten of the most promising candidate biomarkers were selected. This formed the hypothesis that (a combination) of these ten candidate biomarkers is superior to the established biomarkers sPSA and PCA3 to identify patients with Gleason score ≥ 7 prostate cancer upon biopsy. A large multicenter prospective study was conducted within an intended-to-treat cohort, to test the hypothesis. The ten candidate biomarkers were all significantly correlated with Gleason score ≥ 7 prostate cancer. We identified a four gene panel consisting of *HOXC4*, *HOXC6*, *DLX1* and *TDRD1* (QUATTRO) that outperforms sPSA and PCA3 in the Gleason score ≥ 7 prostate cancer diagnosis upon biopsy (AUC 0.78 vs. 0.72 and 0.69 respectively).

Even though QUATTRO was selected on the best achievable clinical diagnostic and prognostic characteristics, we also looked into the tumour biological role of these genes and its possible implications in prostate cancer. The *HOXC4* and *HOXC6* genes belong to the Homeobox family and are located at 12q13.3. *HOXC* over-expression may predispose tumour cells to androgen independence by necessitating adaptation to diminished androgen signalling^{25,26}. *TDRD1* is a male germline-specific gene, located at 10q25.3. It belongs to a large family of Tudor domain containing proteins. Recently, *TDRD1* was identified as a direct *ERG* target gene that is strongly associated with *ERG* over-expression in primary prostate cancer²⁷⁻²⁹. *DLX1* (Distal-less Homeobox 1) is located at 2q32. *DLX1* is involved in the acquisition of epithelial-neuroendocrine differentiation, a characteristic associated with aggressive cancer³⁰. However, so far, the exact function of *HOXC4*, *HOXC6*, *TDRD1* and *DLX1* in prostate cancer is still unknown. Yet, it is possible that these genes are functionally implicated in prostate carcinogenesis.

The relatively high number of urine samples for which we could not run the molecular assays (85/443; 19%) could be considered a limitation of the study. The samples were excluded for reasons of poor RNA quality, using an objective minimally reliable required number of *HPRT* copies. As an ‘in study validation’ we compared clinical diagnostic characteristics of *PCA3* assayed by RT-PCR with that of the FDA approved Progenesa® *PCA3* test. The ROC curves were superimposable, so we considered that as an ‘in study’

validation of the RT-PCR analyses. Also, in the proof of concept study of *PCA3* that was assaying urinary sediments, we could not analyze 15-20% of the samples²³. Therefore, it is most likely that this issue can be solved using whole urine as a substrate rather than urinary sediments.

In this clinical study, we tested our hypothesis formed after the preclinical biomarker discovery study. The next step will be to validate our clinical results in a second independent cohort of men (have been recruited currently). Then, we can test the logistic regression equation model in that independent cohort.

In conclusion, we describe a stepwise development of a four gene-based test that is superior to sPSA and *PCA3* in identifying patients with clinically significant prostate cancer. *HOXC4*, *HOXC6*, *TDRD1* and *DLX1*, referred to as QUATTRO, had independent additional predictive value to sPSA and *PCA3* for predicting Gleason score ≥ 7 prostate cancer diagnosis upon biopsy. QUATTRO added to sPSA could lead to a great reduction in unnecessary biopsies and over treatment.

Acknowledgements

We thank Ms Annelies Hoebers (NovioGendix) for performing ProgenSA *PCA3* testing and Mr Pim Peelen (NovioGendix) for collection of the samples. We thank Mr Ton de Haan (Radboud University Nijmegen Medical Centre) for statistical assistance. We thank PCMM (o3-O-203) for their contribution to this project. Supported in part by grants from EFRO, Ultrasense MR and NovioGendix.

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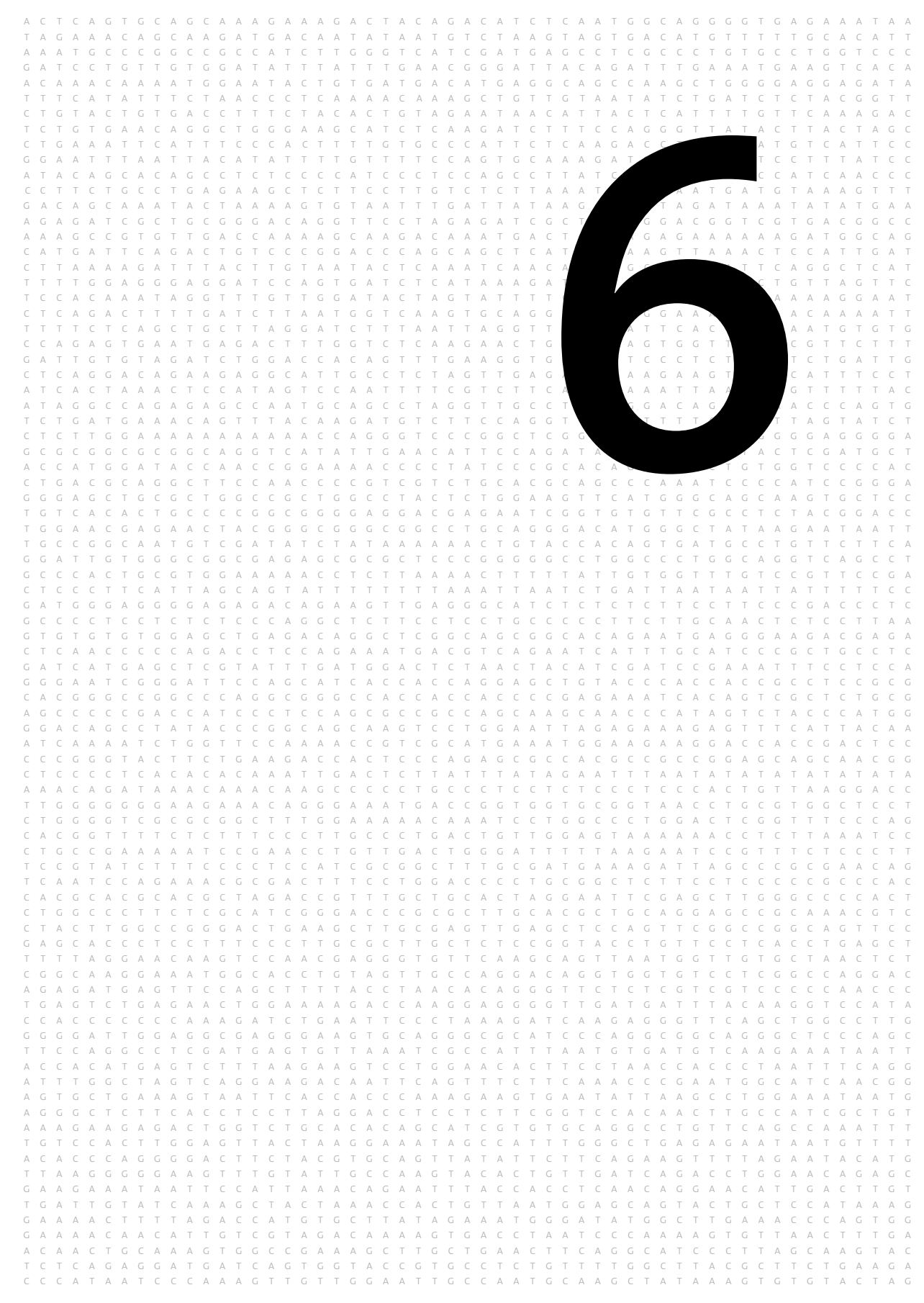
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KLK3, PCA3 and TMPRSS2-ERG expression in the peripheral blood mononuclear cell fraction from castration-resistant prostate cancer patients and response to docetaxel treatment

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Prostate. 2014;74 (12):1222-30

ABSTRACT

Introduction

To monitor systemic disease activity, the potential of circulating tumour cells (CTCs) bears great promise. As surrogate for CTCs we measured KLK3, PCA3 and TMPRSS2-ERG messenger RNA (mRNA) in the peripheral blood mononuclear cell (PBMC) fraction from a castration-resistant prostate cancer (CRPC) patient cohort and three control groups. Moreover, biomarker response to docetaxel treatment was evaluated in the patient group.

Material and Methods

Blood samples from twenty CRPC patients were analyzed at four different time points (prior to docetaxel treatment, at 9 weeks, 27 weeks and 2 months after treatment). Blood was drawn once from three control groups (10 age-matched men, 10 men under 35 years of age, 12 women). All samples were analyzed for KLK3, PCA3 and TMPRSS2-ERG mRNA by using a quantitative nucleic acid amplification assay with gene-specific primers in the complementary DNA synthesis.

Results

At baseline, mRNA for KLK3 was detected in 17 (89%, 95% CI 76-100%), PCA3 in 10 (53%, 95% CI 30-75%) and TMPRSS2-ERG in 7 of 19 evaluable patients (37%, 95% CI 15-59%). In contrast, the blood samples from all 32 healthy volunteers were reproducibly negative for all markers. In response to docetaxel treatment, KLK3 levels decreased in 80% (95% CI 60-100%), PCA3 in 89% (95% CI 68-100%) and TMPRSS2-ERG in 86% (95% CI 60-100%) of patients.

Conclusions

The feasibility of a highly sensitive modified nucleic acid amplification assay to assess KLK3, PCA3 and TMPRSS2-ERG mRNA in the PBMC fraction from CRPC patients was demonstrated. Moreover, response of these markers to systemic treatment was shown.

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INTRODUCTION

Patients with advanced prostate cancer who undergo chemical or surgical castration will ultimately experience a relapse; this state is called castration-resistant prostate cancer (CRPC).The median overall survival of metastatic CRPC patients is 9-13 months¹.Treatment options for CRPC patients are limited. Cytotoxic chemotherapy, immunotherapy, second-line androgen deprivation therapy (the recently FDA approved abiraterone acetate) and novel androgen receptor signalling inhibitors (enzalutamide, ARN 509) are systemic approaches that have been found to prolong survival ²⁻⁷. However, these treatment modalities give only a modest prolongation of overall survival.

The indication and sequence of administration for these new therapies becomes more important and the main issue in the management of CRPC therefore, is the lack of surrogate endpoints for treatment response and survival. Serum PSA, a serine protease secreted by prostate epithelial cells and also known as kallikrein-3 (KLK3), is currently used as biomarker to measure disease burden and to predict treatment efficacy in CRPC patients ⁸⁻¹⁰. For docetaxel therapy, a $\geq 30\%$ sPSA decline within three months of treatment initiation was determined to be the most optimal threshold for the association with overall survival ¹¹. However, there is still a significant proportion of men for whom PSA response does not predict long-term benefit and therefore this threshold did not demonstrate consistent surrogacy for survival and cannot be used as a validated endpoint ^{12,13}. Post-therapy PSA changes are thus unlikely to become a Food and Drug Administration accepted surrogate endpoint for drug registration studies. Accordingly, there is a need to investigate new, reliable and preferably non-invasive methods which can be used as a reliable surrogate endpoint for survival.

In CRPC patients the enumeration of circulating tumour cells (CTCs) in the peripheral blood has been evaluated as a potential prognostic marker and predictor of overall survival ¹⁴⁻¹⁶. The CTC count, defined as favorable (<5 CTCs) or unfavorable (≥ 5 CTCs) at different time points before and after treatment with cytotoxic therapy was the strongest independent predictor of overall survival and would emphasize the CTC enumeration as a potential surrogate marker of therapy response and survival ¹⁴.

Molecular staging may provide more information about systemic disease activity and tumour load. A few studies already described a reverse-transcription (RT) polymerase chain reaction (PCR) to assess PSA mRNA expression in the peripheral blood mononuclear cell (PBMC) fraction ^{17,18}. Moreover, the last decades two new prostate *cancer* specific

biomarkers have been identified; Prostate CAncer gene 3 (PCA3) and the transmembrane protease, serine 2 – ETS gene fusion (TMPRSS2-ERG)^{19,20}. Both markers have been studied intensively as diagnostic prostate cancer markers^{21,22}. Although CTCs are supposed to be fundamental for the establishment of distant metastases, little is known about prostate cancer specific markers in CRPC patients as a surrogate test for CTCs. Nevertheless, a recent study by Danila et al. described an analytic and clinical validation of a prostate cancer-enhanced mRNA detection assay in whole blood as a prognostic biomarker panel for survival. In this study they identified a 5-gene panel, consisting of KLK3, KLK2, HOXB13, GRHL2 and FOXA1, and measured these markers in blood samples from 97 metastatic CRPC patients with progressive disease²³. Results showed that expression of at least two of the five genes was a strong prognostic predictor for survival, comparable with the CellSearch system. Combining the gene panel and the CellSearch resulted in even enhanced power to discriminate between low- and high-risk patients relative to CellSearch alone.

In our study we aimed to assess KLK3, PCA3 and TMPRSS2-ERG mRNA expression in the PBMC fraction of CRPC patients by using a highly sensitive modified reverse transcription-qPCR assay. In addition we evaluated the response of these biomarkers to docetaxel chemotherapy treatment.

MATERIAL AND METHODS

Study design

In this prospective study, blood samples were obtained from twenty-three CRPC patients commencing docetaxel-based chemotherapy at the urology outpatient clinic of our university medical center. Three patients were excluded from biomarker analysis; one patient had an allergic reaction to docetaxel and immediately discontinued treatment after the first dose, one patient continued treatment in another hospital after three cycles and no follow-up data was available, and one patient deceased due to metastasized lung cancer during the study period.

Approval for the study was obtained from the Institutional Review Board in accordance with all medical ethical requirements. After written informed consent was given, blood samples were obtained at four different time points; prior to the first chemotherapy course (baseline), prior to the fourth course (9 weeks), prior to the last (tenth) course (27 weeks) and two months after the last course (37 weeks). As control groups, blood was drawn once from ten healthy age-matched men with no evidence of prostate cancer, ten men aged under 35 and twelve healthy women. Blood samples were collected into two 8

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mL cell preparation tubes (CPT) tubes and processed within two hours. Clinical data (e.g. tumour stage, serum PSA levels, prior treatment, number of docetaxel regimens etcetera) was extracted from the patients records.

Blood processing

Blood collection was coded and collected in two cell preparation tubes (Vacutainer® CPT™ 8mL). The blood processing reported below was according to manufacturer’s instructions. The tubes were centrifuged at room temperature for 20 minutes in a horizontal rotor at 1800 relative centrifugal force (RCF) within two hours of collection. The plasma layer and the cells from both CPT tubes were transferred to one conical centrifuge tube. Phosphate buffered saline (PBS) was added to a final volume of 14 mL, the tube was capped and the cells were mixed by inversion. Subsequently, the tubes were centrifuged for 15 minutes at 4° Celsius (°C) and 1500-1800 RCF.

Supernatant was aspirated and 1mL PBS was added. The cell pellet was resuspended and the suspension was centrifuged at 4°C in a 1.5 mL Safelock tube for 1 minute at 1600 RCF. Supernatant was removed and cell pellets were immediately frozen in liquid nitrogen and stored at -80°C.

RNA extraction

The deep-frozen cell pellets were thawed on ice. Subsequently, RNA was extracted according to the TriPure reagent (Roche, Cat no. 11 667 165 001) guidelines. First 1000 µL TriPure Isolation Reagent was added to the cell pellet and cells were lysed by repetitive pipetting. 200 µL chloroform was added to each sample. The tubes were shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. Subsequently, they were centrifuged at 12,000G for 15 minutes at 2-8°C to separate the solution into three phases. The aqueous upper layer, containing the RNA material, was transferred into a new tube. Isopropanol (500 µL) was added to precipitate the RNA. The samples were incubated at room temperature for 10 minutes. Again samples were centrifuged at 12,000G for 10 minutes and the supernatant was discarded. The RNA pellet was washed in 1000 µL 75-80% ethanol, centrifuged at 7,500G for 5 minutes and supernatant was discarded. The excess of ethanol was removed by air-drying and the RNA pellet was resuspended in RNase-free DEPC-treated water, incubated for 15 minutes at 55-60°C and stored at -80°C.

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Transcript amplification and reverse transcription

The Nanodrop 1000 (Thermo Scientific) was used for both quantification and purity checks of the RNA samples. First-strand cDNA syntheses were done using a modified Qiagen QuantiTect protocol. Ten µg RNA extracted from the mononuclear cells was dissolved in RNase-free water to a volume of 12 µL. Two µL of cDNA wipeout buffer 7x, was added and incubated for 2 minutes at 42°C. Reverse-transcription mastermix containing 1 µL Quantiscript Reverse Transcriptase, 4 µL Quantiscript RT buffer and 1 µL Target-Specific Primers (3 µmol of each primer) was added. The primer sequences (TIB Molbiol) are displayed in Table 1.

Calibration curves with a wide linear dynamic range (10-1,000,000 copies) were generated using serial dilutions of linearized plasmids. Control samples with known template concentrations were used as a reference. Two µL of each cDNA sample, representing approximately 460 µL blood, was amplified in a 20µL PCR reaction, containing 10 µmol of each primer, 2 µmol of hydrolysis probe (TaqMan) and 1x Probe Master mix (Roche). The amplification conditions used were the following: 95°C for 10 minutes followed by 50 cycles at 95°C for 10 seconds, 60°C for 30 seconds and cooling at 40°C for 55 seconds (LightCycler LC480, Roche). LightCycler 480 SW 1.5 software (Roche) was used for determining crossing point values. By extrapolation in the generated calibration curve, crossing point values of the samples were converted to concentrations. The assay performance of the real-time PCR experiments were evaluated during in-study validation. The amplification efficiency of the qPCR assays were determined using the calibration curves. The amplification efficiency was between min. 1.85 and max 2.05. The negative template controls were less than 5 copies.

The reference control samples had an inter- and intra-assay variation <30%. Biomarker mRNA copies per milliliter from each patient are displayed in Table 2.

Table 1 Primer pairs and hydrolysis probe sequences

Gene	GenBank tm	Primer	Position	Used for	Sequence
TMPRSS2	NM_005656	Forward	1-17	qPCR	5'-CCC GAG CTA AGC AGG AG - 3'
ERG	NM_004449	Reverse	315-334	qPCR	5'-GTC CAT AGT CGC TGG AGG AG - 3'
ERG-probe	NM_004449		80-109	qPCR	5'-FAM-TGG TCC TCA CTC ACA ACT GAT AAG GCT TCC-BBQ - 3'
KLK3-F	NM_001648	Forward	74-91	qPCR	5'-CGT GAC GTG GAT TGG TGC - 3'
KLK3-R	NM_001648	Reverse	179-194		5'-GCC GCA GAC TGC CCT G - 3'
KLK3-A	NM_001648	Reverse	187-170	qPCR	5'-ACTGCCCTGCCACGAGAG - 3'
KLK3-probe	NM_001648		97-122	qPCR	5'-610-CCC TCA TCC TGT CTC GGA TTG TGG GA- BBQ - 3'
PCA3-F	AF_103907	Forward	24-47	qPCR	5'-GAA GCT GGC ATC AGA AAA ACA GAG - 3'
PCA3-R	AF_103907	Reverse	334-311	qPCR	5'-AGA TGT GTG GCC TCA GAT GGT AAA - 3'
PCA3-probe	AF_103907		90-113	qPCR	5'-FAM-TGC ATG GTG GGA AGG ACC TGA TGA-BBQ - 3'

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Table 2 Biomarker data per patient per timepoint

Biomarker	sPSA (ng/mL)				KLK3 (copies/ml)				PCA3 (copies/ml)				TMPRSS2-ERG (copies/ml)			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	7.4	1.2	0.7	1.9	36	0	0	116	0	0	0	49	7208	0	0	17273
2	616	214	248	635	325	184	540	4082	35	0	0	0	0	0	0	0
3	115	482	57.1	115	23643	1248	0	165	19543	999	57	50	35206	0	0	0
4	28.5	9.6	1.5	2.2	97	0	0	34	0	0	0	0	0	0	0	0
5	70	2.2	41.5	14.7	480	0	2801	244	57	0	442	96	11815	0	21102	12597
6	31.5	10.2	7.7	6	66	0	0	0	0	0	0	0	0	0	0	0
7	357	829	642	897	1735	5704		786	656	1099		372	19572	35205		51388
8	43.2	65.8	71	177	25	0	0	99	0	0	0	0	0	0	0	0
9	179	281	15.3		5255	8713	1637		0	0	0	0	0	0	0	
10	36.3	9.9	11.6	95.1	0	0	0	248	0	0	0	0	0	0	0	10297
11	9.9	1.4	14.2		33	78	100		0	0	0	0	0	0	0	
12	58.8	51.1	28	530	1064	120	418	1735	133	51	140	322	28445	0	21856	34761
13	168	5.6	3.9	5.5	5243	0	477	345	101	0	0	0	55198	0	15882	16360
14	81.5	42.7	7.4	57.7		0	0	39		53	0	34		0	0	0
15	189	272	99.7	76.4	208	0	0	0	50	0	0	0	14539	0	0	0
16	105	70.6	123		81	18	159		88	54	158		0	0	0	
17	38.2	17	26.4		104	133	178		168	146	108		0	0	0	
18	50	31.5	15	49	54	0	0	0	0	0	0	0	0	0	0	0
19	161	23.1	1.3	4.7	0	0	0	0	0	0	0	0	0	0	0	0
20	470	176	50	77.7	3762	187	70	237	165	0	46	46	0	0	0	0
Mean	141	130	73	172	2222	819	336	508	1105	120	50	61	9052	1760	3097	8917
Median	76	37	21	67	104	0	0	141	35	0	0	0	0	0	0	0
Minimum	7.4	1.2	0.7	1.9	0	0	0	0	0	0	0	0	0	0	0	0
Maximum	616	829	642	897	23643	8713	2801	4082	19543	1099	442	372	55198	35205	21856	51388

Abbreviations: sPSA, serum prostate specific antigen; KLK3, kallikrein-3; PCA3, prostate cancer gene 3

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Statistical analysis

Since the study was of exploratory nature, statistics were mainly descriptive. RT-PCR was performed twice for each sample to test for reproducibility and biomarkers were classified positive if ≥ 20 copies per PCR reaction were present. The association between biomarker expression presence and survival outcome was assessed using the log rank test and a p -value < 0.05 was considered statistically significant.

RESULTS

Peripheral blood samples from 20 CRPC patients commencing docetaxel treatment, collected at four different time points (i.e. baseline, 9 weeks, 27 weeks and 37 weeks), were analyzed. One baseline sample was not suitable for processing and one sample from the third time point was lost during transportation. Four patients discontinued treatment prematurely, were discharged from further follow-up at our hospital and were consequently not available for the fourth time point measurement. Therefore, 19 samples (95%) at baseline, 20 samples (100%) at the second time point, 19 samples (95%) at the third time point and 16 samples (80%) at the last time point were available for biomarker evaluation.

The patient characteristics are presented in Table 3. The mean number of administered docetaxel regimens was 8 (range 2-10 cycles), 8 patients (40%) completed 10 cycles of docetaxel treatment and 12 patients (60%) discontinued at an earlier stage. The main reason for treatment discontinuation was serum PSA rise (58%).

Baseline biomarker expression

CRPC patient RT-PCR biomarker data, together with serum PSA levels, are displayed in Table 2. The median PSA level of the chemotherapy-naïve CRPC patients was 75.8 ng/mL. KLK3 mRNA expression was detected in 17 of the 19 evaluable CRPC patient samples at baseline (89%, 95% CI 76-100%), PCA3 in 10 patients (53%, 95% CI 30-75%) and TMPRSS2-ERG in 7 patients (37%, 95% CI 15-59%). Eleven patients (58%, 95% CI 36-80%) were either PCA3 or TMPRSS2-ERG positive and in 6 patients (32%, 95% CI 11-53%) both mRNAs were detected.

The reproducibility for mRNA expression at baseline in a second RT-PCR was 100% for KLK3 and TMPRSS2-ERG and 84% for PCA3. This means that in all patients KLK3 and TMPRSS2-ERG expression in the first test, showed the same results in a second (validity) test of the same blood samples. For PCA3 16% of the test samples showed discrepancy in expression between the first and the second test.

Table 3 Patient characteristics

	CRPC patients n=20 No. (%) / median (IQR)	Age-matched men n=10 No. (%) / median (IQR)	Men age <35 n=10 No. (%) / median (IQR)	Women n=12 No. (%) / median (IQR)
Age, years	69 (62-72)	68 (63-69)	27 (24-31)	57 (45-64)
Metastasis status				
Primary M+ diagnosis	9 (45)	-	-	-
M+ after primary treatment	11 (55)	-	-	-
Primary treatment				
Radical prostatectomy	6 (30)	-	-	-
External radiation therapy	2 (10)	-	-	-
Brachytherapy	3 (15)	-	-	-
No primary treatment	9 (45)	-	-	-
Prior systemic therapy				
Androgen deprivation	20 (100)	-	-	-
Chemotherapy	0	-	-	-
Number of docetaxel regimens	8.5 (5-10)	-	-	-
Samples available for analysis				
Baseline	19 (95)	10 (100)	10 (100)	12 (100)
2 nd timepoint	20 (100)	-	-	-
3 rd timepoint	19 (95)	-	-	-
16 (80)		-	-	-
sPSA, ng/mL				
Baseline PSA	76 (37-176)	-	-	-
2 nd timepoint	37 (10-205)	-	-	-
3 rd timepoint	21 (7-68)	-	-	-
4 th timepoint	67 (6-162)	-	-	-
sPSA nadir, ng/mL	15 (2-55)	-	-	-
Follow-up, months	18 (13-21)	-	-	-
Patients died	13 (65)	-	-	-

Abbreviations: CRPC, castration-resistant prostate cancer; no., number; IQR, interquartile range; sPSA, serum prostate specific antigen

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The median copy numbers per milliliter blood for positive KLK3 mRNA was 208 (interquartile range [IQR] 60-2749), for positive PCA3 mRNA this was 117 (IQR 55-290) and for positive TMPRSS2-ERG mRNA 19572 (IQR 11815-35206). In contrast, the blood samples from all 32 healthy volunteers were negative for KLK3, PCA3 and TMPRSS2-ERG mRNA (Table 3).

Biomarker expression and treatment response

Seven patients (35%) underwent all 10 docetaxel cycles and had evaluable samples at all 4 time points. The biomarker and serum PSA levels for this group at all time points are displayed as a bar graph in Figure 1.

Seventeen patients (85%) had evaluable samples at both baseline and after 9 weeks of treatment and underwent at least 4 treatment cycles. Serum PSA levels decreased in 12 patients (71%, 95% CI 49-92%) and the median decrease in PSA was 51%. KLK3 decreased in 12 of 15 positive patients (80%, 95% CI 60-100%), PCA3 in 8 of 9 positive patients (89%, 95% CI 68-100%), and TMPRSS2-ERG in 6 of 7 positive patients (86%, 95% CI 60-100%). Of the 12 patients that showed a decrease in KLK3 expression, 9 patients (75%, 95% CI 51-100%) also showed a decrease in serum PSA level. From the other 3 patients, that showed an increase in PSA levels, 2 were PCA3 and TMPRSS2-ERG positive at baseline, and in accordance with KLK3, a decrease in expression level was detected at 9 weeks. Moreover, 2 of the 3 patients that had a KLK3 increase at 9 weeks also showed serum PSA increase at 9 weeks.

In addition, at the last time point, most patients had increased serum PSA and biomarker expression levels compared to one of the previous measure points during treatment. Thirteen of 16 patients (81%, 95% CI 62-100%) had a serum PSA increase. Moreover, an increase was seen for KLK3 in 9 of 15 patients (60%, 95% CI 35-85%), for PCA3 in 5 of 9 (56%, 95% CI 23-88%) and for TMPRSS2-ERG in 5 of 7 (71%, 95% CI 38-100%). The number of patients with positive biomarker expression at the different time points is presented in Table 4.

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Table 4 Number of patients (%) with positive biomarker expression (≥ 20 copies per PCR reaction)

	CRPC patients				Age-matched men	Men < 35	Women
Time point	1	2	3	4			
No. patients	19	20	19	16	10	10	12
KLK3	17 (89)	9 (45)	9 (47)	12 (75)	0	0	0
PCA3	10 (53)	6 (30)	6 (32)	7 (44)	0	0	0
TMPRSS2-ERG	7 (37)	1 (5)	3 (16)	6 (38)	0	0	0

Abbreviations: CRPC, castration-resistant prostate cancer; KLK3, kallikrein-3; PCA3, prostate cancer gene 3

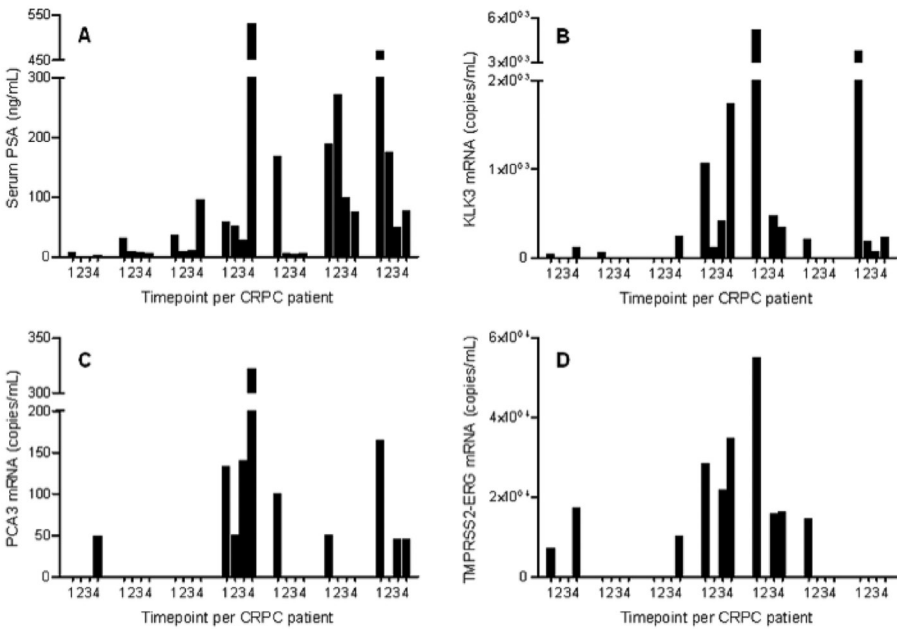


Figure 1 Biomarker expression for serum PSA (A), KLK3 (B), PCA3 (C) and TMPRSS2-ERG (D) at all time points in response to docetaxel treatment for seven CRPC patients who underwent ten treatment cycles (1=baseline, 2=9 weeks of treatment, 3=27 weeks of treatment, 4=2 months after last cycle).

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Prognostic value of biomarker expression

To assess PCA3 and TMPRSS2-ERG as prognostic factors, survival information was used to calculate a correlation for baseline biomarker positive patients and baseline biomarker negative patients in relation to overall survival. The mean follow-up was 18.5 months (range from 10.7 to 33.1 months). Patients with negative baseline PCA3 expression had no statistically significant longer median survival (19.4 months [95% CI 13.9-24.8]) compared to patients with positive baseline PCA3 expression (15.0 months [95% CI 5.3-24.7], log rank $p=0.907$). Median survival for TMPRSS2-ERG negative (16.2 months [95% CI 10.1-22.3]) compared to the TMPRSS2-ERG positive patients (26.9 months [95% CI 15.7-38.2]) showed a clear trend towards a longer survival ($p=0.175$).

DISCUSSION

In the present study we used highly sensitive modified reverse transcription-qPCR assays to detect prostate *tissue* specific KLK3 mRNA and prostate *cancer* specific PCA3 and TMPRSS2-ERG mRNAs in the PBMC fraction of a CRPC patient cohort and three control groups consisting of healthy-volunteers. The use of gene-specific primers instead of random or mixed primers for the first-strand complementary DNA (cDNA) synthesis reaction, resulted in a high analytical sensitivity of biomarker detection. KLK3 was detected in 89% of CRPC patient samples at baseline (17 of 19). Since KLK3 mRNA is only produced by prostate cells, the detection in the PBMC fraction is indicative for circulating prostate cells ²⁴. In turn, circulating prostate cells suggest a form of metastatic disease activity. However, earlier studies demonstrated the presence of KLK3 expression in peripheral blood samples from patients with clinically localized prostate cancer (T1-2) ^{17,18}. Accordingly, this suggests that in prostate cancer, prostate cells are released into the bloodstream regardless of the disease stage, although in these studies expression was found to be higher in metastatic prostate cancer patients. Our study did not include a group of localized prostate cancer patients, nevertheless, the group of patients without prostate cancer were negative for the biomarkers tested, indicating that KLK3 mRNA expression in the PBMC fraction is specific for prostate cancer.

The rate of detectable KLK3 mRNA in this study is higher than described in earlier studies and might be explained by the use of gene-specific primers in the cDNA synthesis. These primers specifically prime the reverse transcription activity to a specific target mRNA, instead of priming all mRNAs in the mixture, and thereby enhance analytical sensitivity. In a study by Ghossein et al. consisting of 76 metastatic prostate cancer patients, KLK3 was detected in only 34% and another study reported positive KLK3 mRNA expression

in 14 of 18 metastatic patients (78%)^{17,18}. A more recent study included 180 patients with localized prostate cancer, 76 metastatic CRPC patients and 19 healthy volunteers. KLK3 mRNA was positive in 27 of 76 CRPC patients (36%) and in 15 of 180 patients (8%) with localized disease²⁵.

Since KLK3 is a prostate *tissue* specific gene more than a *cancer* specific gene, the cancer specific markers PCA3 and TMPRSS2-ERG were also assayed. At baseline TMPRSS2-ERG was detected in 37% of the patients (7 of 19). This detection rate is in accordance with prior studies in which the fusion was detected in prostate cancer specimen and urine from prostate cancer patients^{20,26,27}. In a study by Danila et al. TMPRSS2-ERG expression in circulating tumour cells was assessed in 41 CRPC patients and an equal detection frequency of 37% was described²⁸.

PCA3 mRNA was detected in 53% of CRPC patients. To date, only two studies have been performed analyzing PCA3 transcripts in blood samples from prostate cancer patients. Jost et al. described an automated sample processing method, using an immunomagnetic fractionation procedure to enrich CTCs from peripheral blood specimens and subsequently performed molecular assays for KLK3, PCA3 and TMPRSS2-ERG. However, PCA3 was detected in only 5 of 33 patients (15%) and TMPRSS2-ERG in 4 of 33 patients (12%)²⁹. In another study by Väänänen et al. mRNA levels were analyzed in peripheral blood samples from 67 prostate cancer patients, including 9 patients with metastatic prostate cancer³⁰. Only 2 of these 9 patients (22%) showed PCA3 mRNA above the limit of quantification. This low number of PCA3 positive patients can be explained by the use of random primers for cDNA synthesis. In contrast, the use of gene-specific primers together with increasing the template volume for the PCR reaction in our study enhanced biomarker analytical sensitivity and resulted in a higher fraction of PCA3 positive patients compared to assays using random primers (*data not shown*). To internally validate this method and assess test reproducibility, RT-PCR assays were performed twice on each sample. In total, three biomarker mRNAs were analyzed in 106 samples, i.e. 636 assays were carried out. In the second (validity) assay, biomarker expression was reproducible with respect to the first assay in 98%. Only 13 patient assays (2%) appeared not to be reproducible (*data not shown*). This data demonstrates the high reliability of the assays.

Although this was an exploratory study, and thus the patient group was too small to make a valid statement on the prognostic value of these biomarker expression status, the biomarkers were measured longitudinally during treatment to assess a biomarker treatment response. In most patients with positive biomarker expression at baseline a decrease in biomarker expression was detected after three cycles of docetaxel treatment (80-89%). Serum PSA levels decreased in 71% of patients as response to three cycles of

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docetaxel, however, a 75% conformity between KLK3 expression and serum PSA response was detected. Furthermore, at the last measure point biomarker expression increased in most patients (56-71%), suggesting a reflection of an increasing disease burden after discontinuation of the treatment. Although this study was not designed to evaluate the prognostic value of these markers, results imply that these biomarkers may be used as a prognostic tool in CRPC patients and/or to measure therapy effect, however, validation studies on this endpoint are ongoing.

CONCLUSIONS

To our knowledge this was the first study that combined detection of KLK3, PCA3 and TMPRSS2-ERG expression assays in the PBMC fraction from CRPC patients and additionally assessed expression levels over time in relation to systemic treatment. The results demonstrate the feasibility of a highly sensitive reverse transcription qPCR assay, using gene-specific primers in the cDNA synthesis, as a nucleic acid test to detect biomarkers in the PBMC fraction. Since no valid prognostic value could be demonstrated for the concerning biomarkers, larger prospective clinical studies need to shed more light on the relevance of these findings.

Acknowledgements

This work was partly supported by a grant from EFRO, ULTRASENSE MR (EFRO: 2010-013177). The patients that participated in this study are greatly acknowledged for their contribution.

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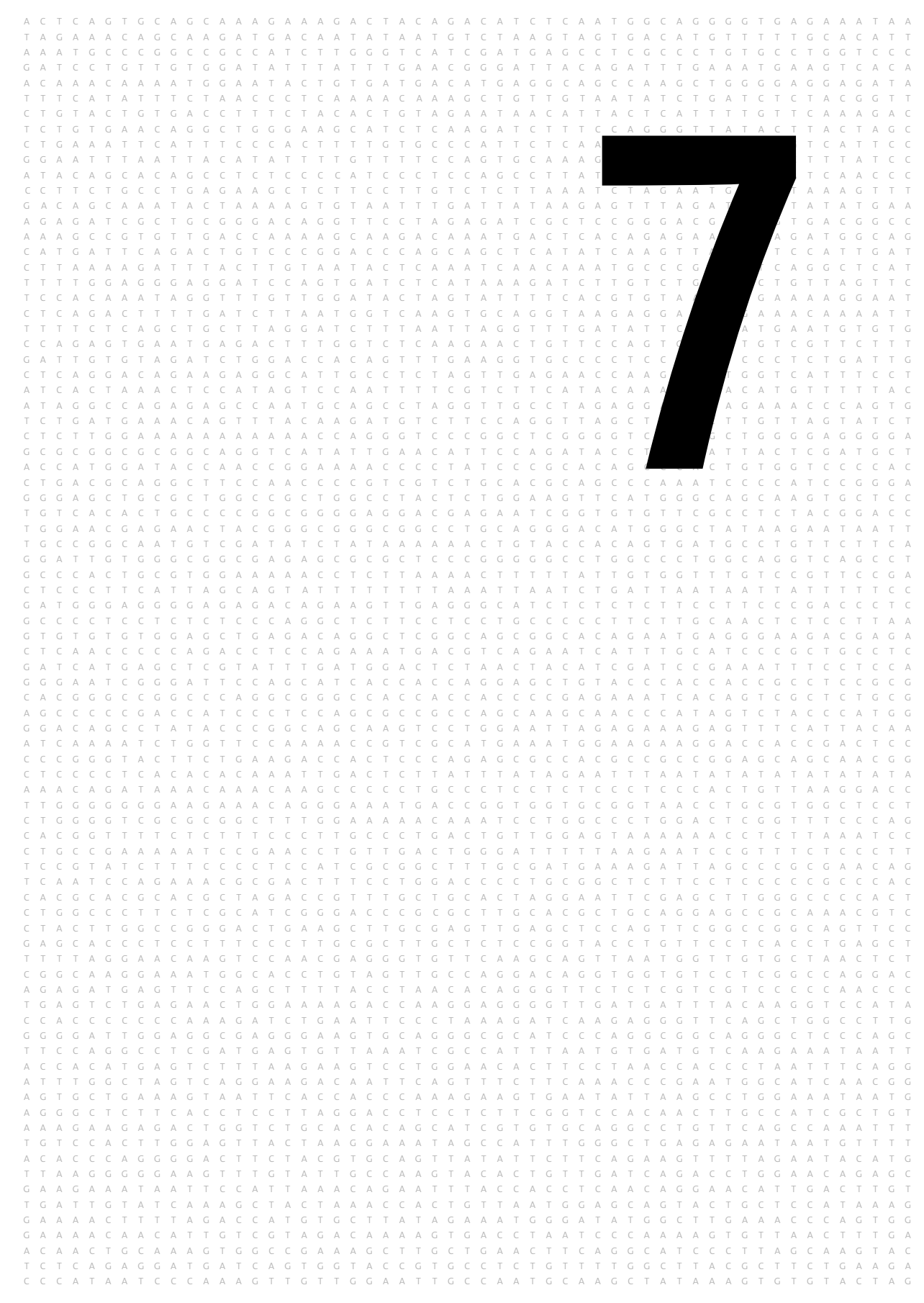
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Discussion and future perspectives

The current landscape of prostate cancer diagnosis

Serum PSA is currently the only widely used biomarker for the diagnosis prostate cancer. As discussed previously in this thesis, the use of PSA has several drawbacks. In brief, PSA has a low specificity (25-40% for PSA levels 4.0-10.0 ng/mL), resulting in a high negative biopsy rate^{1,2}. Also, widespread PSA testing leads to the diagnosis of clinically insignificant tumours, resulting in potential overtreatment (see introduction). Therefore, highly specific and prognostic biomarkers are urgently needed.

In this search for more specific biomarkers for prostate cancer, PCA 3 and TMPRSS2-ERG gene fusion transcripts have been identified as promising urinary novel biomarkers^{3,4}. The ProgenSA PCA3-test is FDA approved since 2012 and commercially available to aid in the decision of taking (repeat) prostate biopsies. However, the prognostic value of PCA3 seems limited⁵⁻⁸. TMPRSS2-ERG gene fusion transcripts are highly specific for prostate cancer, but only present in approximately half of the prostate cancer patients. This urine test (not yet commercially available) has a sensitivity of 37% and specificity of 93% for the prediction of prostate cancer upon prostate biopsy⁹. The prognostic value of this urine test is still under investigation.

Limitations of TRUS-guided biopsies and histopathology as the gold standard

A major limitation for the identification of new prostate cancer biomarkers, is the lack of a true gold standard. The current gold standard is the histopathological evaluation of prostate biopsies. However, systematic random biopsy is prone to undersampling (15-25% cancers missed on first biopsy)¹⁰. In other words, the perfect marker (with 100% accuracy) can only reach a maximum specificity of 92% due to the lack of a robust gold standard. In example: a group of 100 patients, 30 of these patients will have prostate cancer, and 6 patients with prostate cancer will be missed on biopsy (20%), despite a positive marker test; hence, specificity $70/76=92\%$. Furthermore, an underestimation of the Gleason grade of up to 46% of cases has been reported¹¹, and the interobserver variability for Gleason score is extremely high (53%)¹². It must be noted that of course this is not only a major limitation for the identification of new biomarkers, above all, this is a major limitation in the clinical arena. A man with a Gleason 6 prostate cancer diagnosis might qualify for Active Surveillance, whereas a man with a Gleason 7 prostate cancer must be advised to undergo treatment. Quite a difference.

Improving the gold standard in prostate cancer diagnosis; multiparametric MRI

Another novelty in the diagnostic process of prostate cancer is the multiparametric MRI, which is being used increasingly. If read by an experienced radiologist, this technique

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has a high accuracy (80-90%) to detect prostate cancer¹³⁻¹⁶. MRI is a promising technique to improve the current gold standard. MR-guided biopsies are claimed to increase the detection of (significant) prostate cancer and reduce understaging¹⁷⁻²⁰. Thus, with MR-guided biopsy, the gold standard is assumed to be improved compared to TRUS-guided biopsies. Consequently, MR-guided biopsies will be important to include in all future biomarker studies. With MR-guided biopsy outcome as an endpoint, the actual presence of prostate cancer will be reflected better. In other words, the true performance of the (candidate) biomarker will be assessed more accurately.

Due to additional expenses and limited availability, the routine application of MR-guided biopsy is not (yet) feasible. Thus, clinical parameters (i.e. biomarkers) can be used to select patients requiring MR(-guided biopsy). As evaluated in our explorative study in Chapter 3, PCA3 bears promise as a tool for selecting patients requiring MR(-guided biopsy). The value of QUATTRO to predict MRI outcome will have to be evaluated in the future. As we have shown QUATTRO has prognostic value, it is likely to be more suitable for selecting patients for MR(-guided biopsy). This will have to be evaluated in future studies.

Urine as substrate for prediction of biopsy outcome

Urine can be easily obtained in a non-invasive manner, this makes it a promising substrate for routine testing. Various components of the urine can be used: whole urine or urinary sediments. The ProgenSA PCA3-test is based on whole urine as a substrate. However, the first experimental PCA3-test and the QUATTRO test in this thesis was performed on urinary sediments. The advantage of using urinary sediments may be that all cells and cell fragments in the sample are used, in contrast to whole urine where only part of the total urine sample (2.5ml) is analyzed. On the other hand, the advantage of whole urine samples is a more simple processing procedure and samples are more stable during shipment. Thus, for clinical purposes, whole urine samples are often preferred.

Since 2004, exosomes are studied intensively as an alternative substrate. Exosomes are small tissue-derived vesicles which contain a wide variety of proteins and RNA's that represent their tissue of origin²¹⁻²⁴. Exosomal RNA is assumed to be protected by the vesicles and therefore better preserved compared to RNA within whole cells. This is still under investigation. The differences between the use of whole urine , urinary cell pellets and -exosomes is still not fully understood, albeit that clinical performance PCA3 and TMPRSS2-ERG gene fusion tests on cell pellets and whole urine, was similar.

Advanced prostate cancer; Surrogates for circulating tumour cells

Despite an increase in treatment options for castrate resistant prostate cancer (CRPC) patients, therapeutic outcome is still poor. Median overall survival of metastatic CRPC patients is 9-13 months²⁵. Treatment with cytotoxic chemotherapy, immunotherapy, second-line androgen deprivation therapy (i.e. abiraterone) or ARSIs (i.e. enzalutamide) only give a modest prolongation of the overall survival²⁶⁻³⁰.

The main issue with the development of new therapies for CRPC patients is the lack of qualified surrogate endpoints for survival³¹⁻³³. There is a large proportion of patients for whom PSA response does not predict long-term benefit^{31,32}. Therefore, post therapy changes in PSA are unlikely to become an FDA-accepted surrogate end point for drug registration studies. Reliable surrogate end points are being investigated in order to reduce the time required to assess the efficacy of a new treatment and appropriate selection of patients for clinical trials. Circulating tumour cells are currently the most accurate and independent predictors of overall survival in CRPC patients³⁴.

Furthermore, circulating tumour cell analysis would be a great step forward to personalized cancer therapy. As it could enable us to molecularly characterize a patients cancer at different time points in treatment. The ability to take a 'liquid biopsy' could potentially assist in a better therapeutic strategy and in guiding earlier discontinuation of ineffective treatment.

Prostate cancer specific biomarkers measured in peripheral blood samples might serve as a reliable method of detecting circulating tumour cells. In our explorative study (Chapter 6) we demonstrated the feasibility to detect PCA3 and TMPRSS2-ERG in peripheral blood samples. This is an important first step in the evaluation of the value of prostate cancer specific biomarkers (i.e. PCA3, TMPRSS2-ERG and QUATTRO) in peripheral blood samples as a surrogate end point of overall survival. A large prospective clinical study will be the next step.

Biomarkers as a therapeutic target

Probably a longer way ahead of us, but prostate cancer biomarkers might also have potential as a therapeutic target. Prostate cancer specific biomarkers provide important understanding of factors influencing the aggressiveness of prostate cancer, thereby could lead to the development of novel therapies. The classic example of a gene fusion implicated in cancer development is BCR:ABL fusion in patients with chronic myelogenous leukemia. This discovery has been revolutionary as it has lead to the development of imatinib³⁵. This is an inhibitor of the BCR:ABL gene fusion product which transformed the previously fatal leukemia into a manageable chronic disease for many patients. The FDA

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has approved imatinib as first line treatment for newly diagnosed CML in 2002. Another example of a biomarker with therapeutic importance is HER2/neu. Overexpression of HER2 (20-25% of breast cancer patients), is associated with poor clinical outcomes, resistance to Tamoxifen therapy and relative sensitivity to chemotherapy regimens³⁶. Targeted therapy with HER2-targeted monoclonal antibodies Trastuzumab (Herceptin) has been developed and improvements in the overall survival has been demonstrated^{37,38}.

Quality of translational biomarker research

In the worldwide search for novel diagnostic and prognostic biomarkers for prostate cancer, many tumour markers have been proposed. The number of articles published on this subject has increased substantially in the last decade. However, PSA (and derivatives), PCA3 and circulating tumour cells are still the only ones used in clinical practice. Many published results on novel prostate cancer biomarkers appear not reproducible in subsequent studies and thus will never attain the FDA approved status. Where a double-blind randomized placebo controlled trial is the gold standard for therapeutic studies, biomarker studies are not regulated by clear guidelines. These studies often suffer poor study design, lack methodological quality and standardized assays, and information on key elements of design and analysis are often not reported. To improve the quality of diagnostic studies, the STARD (STAndards for Reporting of Diagnostic accuracy) statement was developed by a group of scientists and editors in 2003³⁹. It consists of a checklist of 25 items and flow diagram that authors can use to ensure that all relevant information is present. In addition, the REMARK guidelines (Reporting Recommendations for Tumor Marker Prognostic Studies) were published in 2005⁴⁰. These are guidelines for transparent and complete reporting of studies, so that poor studies can be better identified. These initiatives are important steps forward in improving the quality of tumour marker studies, but further improvement of future studies is warranted. Other future improvement includes the use of an secured database with audit-trail, so that results cannot be manipulated after analysis.

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Future studies

Research is making small steps forward, thus every thesis ends with new proposals for future studies.

QUATTRO is a promising prognostic four gene-based test that could improve the diagnostic process of prostate cancer significantly. Yet, our results will have to be validated in a second independent cohort of men. MR-guided biopsy outcome should be used as the endpoint in this study, in order to improve the gold standard (as discussed earlier). In addition, QUATTRO will have to be evaluated in whole urine specimen (instead of urinary sediments), as this might be a more suitable substrate for clinical use. Whole urine specimen are more stable during shipment, and the processing procedure is more simple.

For men on Active surveillance, novel biomarkers might be of help to stratify better the risk of having clinical significant prostate cancer. A recent study evaluated the potential of PCA3 and TMPRSS2-ERG to monitor patients on active surveillance⁴¹. PCA3 and TMPRSS2-ERG were correlated with tumour volume and presence of high grade prostate cancer. This will have to be further studied in large prospective trials, in which also the potential of QUATTRO will have to be evaluated. As QUATTRO may possibly be a test with more prognostic value, and might be more suitable for this purpose than PCA3 and TMPRSS2-ERG.

In future, urine biomarkers might shift to first line medicine, the general practitioner. A proposal for a future study would be: all patients at the general practitioner with PSA levels ≥ 3.0 will undergo a urine test. If urinary biomarkers are low, patients will stay for follow up at the general practitioner. If urinary biomarkers are elevated, the patient is sent to the urologist for an MRI, to stratify patients for (MR-guided) prostate biopsies.

With the currently used biomarker serum PSA, population based screening for prostate cancer is not beneficial. However, with a combination of these cancer-specific urinary biomarkers, PSA and MRI population screening for prostate cancer could be effective. This would have to be evaluated in a large prospective study, in accordance with the previous ERSPC studies.

To further study the potential of prostate cancer biomarkers as a reliable method of detecting circulating tumour cells, a large prospective multicenter trial is needed. The study design of our explorative study in Chapter 6 can be used, only then in a large multicenter setting. Also, it would be very interesting to include QUATTRO in this study.

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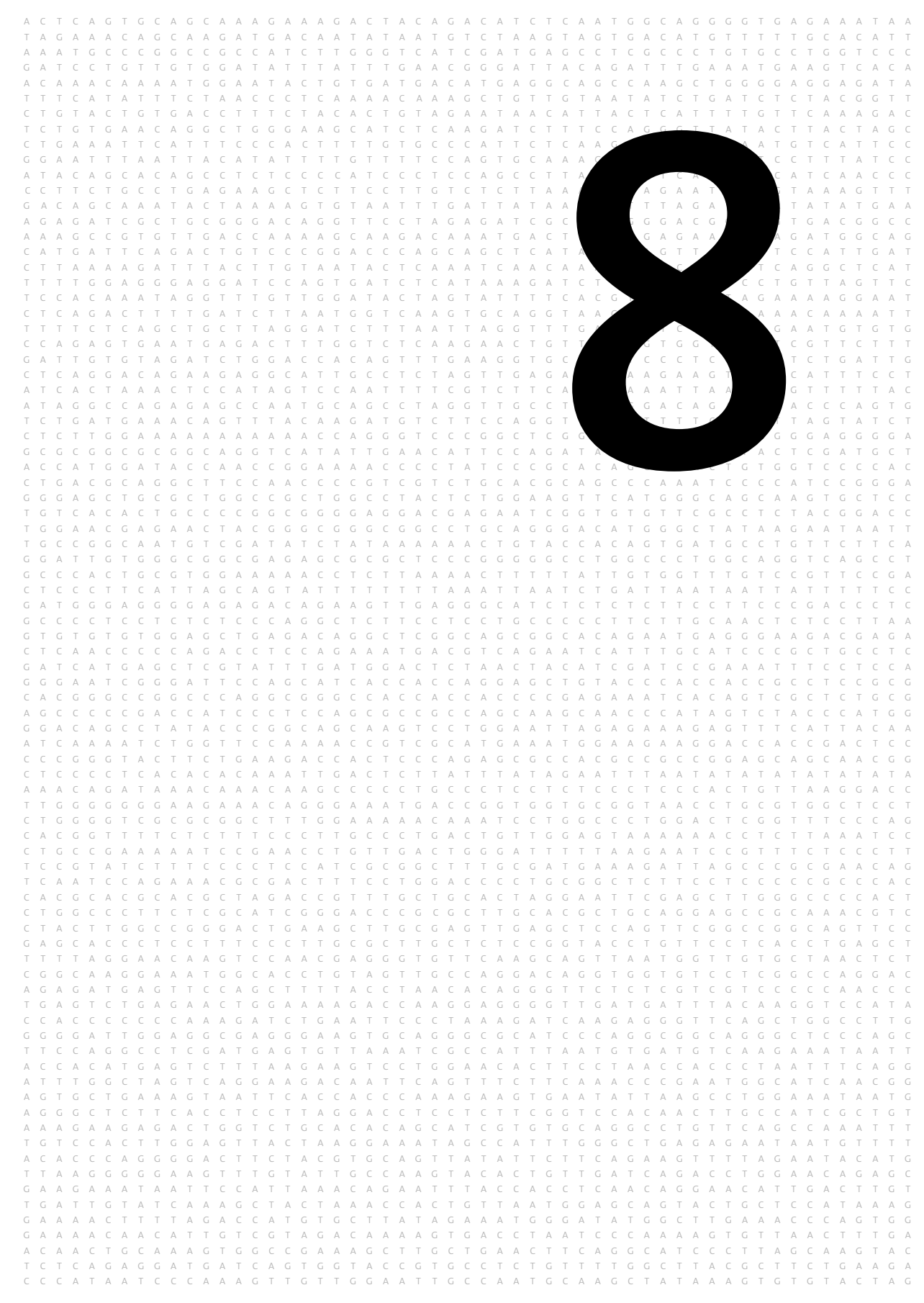
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Summary

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Prostate cancer is the second most commonly diagnosed cancer in men. The gold standard for the diagnosis of prostate cancer is based on the histopathological evaluation of prostate biopsies, an invasive procedure with significant morbidity. Localized prostate cancer often does not present with symptoms, therefore, the selection of men qualifying for prostate biopsies relies on serum PSA (prostate specific antigen) testing and digital rectal examination. **Chapter 1** presents the general introduction and the outline of this thesis.

Chapter 2 gives a short history of prostate cancer biomarkers, an overview of the established prostate cancer biomarkers PSA and PCA3; and promising novel prostate cancer biomarkers (tissue markers, blood markers and urine markers): TMPSS2-ERG, Ki-67/MIB1-Labeling Index, PTEN, E-cadherin, EZH2, neuroendocrine phenotype, microRNAs and circulating tumor cells (CTCs).

A urine test is a non invasive method to obtain prostate (cancer) cells, which makes it suitable for clinical purposes. A digital rectal examination is performed before collecting the urine sample to mobilize prostatic cells toward the prostatic urethra, which are flushed out with the first-voided urine. The ProgenSA PCA3 test is FDA approved and clinically available. PCA3 is highly overexpressed in prostate tumours compared to the adjacent benign prostate tissues. The ProgenSA PCA3 test is currently used to aid in the decision to take (repeat) biopsies. Furthermore, the multiparametric 3 tesla MRI is being used increasingly in the diagnostic process of prostate cancer. It has a high accuracy to detect prostate cancer. However, due to additional expenses and limited availability compared to the conventional TRUS-guided biopsy, the routine application of MRI-guided biopsy is not yet feasible. An additional, cheaper and more practical test to select patients who require multiparametric MRI, and potentially subsequent MR-guided biopsy, would be valuable. In **chapter 3**, we evaluated the clinical value of the ProgenSA PCA3 test: its predictive value for biopsy outcome, Gleason score and MRI outcome. This was evaluated in a retrospective study of 591 men who underwent a ProgenSA PCA3 test at the Radboud University Nijmegen Medical Centre. Prostate biopsies were performed in 290 men, a multiparametric MRI of the prostate was performed in 163/591 men. The PCA3 score was highly predictive for biopsy outcome ($p < 0.001$), there was no correlation with Gleason score upon biopsy ($p = 0.194$). The PCA3 score of patients with a suspicious region for prostate cancer on MRI was significantly higher ($p < 0.001$) than in patients with no suspicious region on MRI (52 vs. 21). This suggests that PCA3 could be used to select patients that require MRI.

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In the ongoing search for novel prostate cancer biomarkers, TMPRSS2-ERG gene fusion transcripts have been identified as a promising novel urinary biomarker. In prostate cancer, TMPRSS can be fused to ERG. These TMPRSS2-ERG gene fusions are specific for prostate cancer and are present in approximately half of the prostate cancer patients. This urine test has a sensitivity of 37% and specificity of 93% for the prediction of prostate cancer upon prostate biopsy. Considering the heterogeneous character of the disease, the use of a biomarker panel can further improve the diagnostic accuracy. In **chapter 4**, we present the results of a large prospective study that evaluated the diagnostic and prognostic value of ProgenSA PCA3 and TMPRSS2-ERG gene fusions for prostate cancer, as individual markers and as a biomarker panel. Both PCA3 and TMPRSS2-ERG had significant additional predictive value ($p<0.001$ and resp. $p=0.002$) to the ERSPC risk calculator parameters (serum PSA, DRE, TRUS and prostate volume). The AUC increased from 0.799 (ERSPC risk calculator), to 0.833 (ERSPC risk calculator + PCA3), to 0.842 (ERSPC risk calculator + PCA3 + TMPRSS2-ERG) to predict prostate cancer. Sensitivity of PCA3 increased from 68% to 76% when combined with TMPRSS2-ERG. TMPRSS2-ERG had significant additional predictive value to the ERSPC risk calculator to predict biopsy Gleason score ($p<0.001$) and clinical tumor stage ($p=0.023$), whereas PCA3 did not. In conclusion, this study shows that TMPRSS2-ERG had independent additional predictive value to PCA3 and the ERSPC risk calculator parameters for predicting prostate cancer. Furthermore, TMPRSS2-ERG had prognostic value, whereas PCA3 did not.

ProgenSA PCA3 aids in the diagnosis of prostate cancer. However, its prognostic value seems limited. Therefore, biomarkers that can be used to identify patients with clinically significant disease are urgently needed. To address this unmet need, the stepwise development of a four gene based urinary test is described in **chapter 5**. Using microarray analysis on snap frozen tissue, 46 candidate biomarkers were selected. Validation using Taqman low density arrays (TLDA), identified the ten most promising biomarkers (and). This led to the hypothesis that a combination of these ten biomarkers is superior to sPSA and PCA3 to identify patients with Gleason score ≥ 7 prostate cancer. This hypothesis was tested in a prospective multicenter study on urine specimens of 358 men undergoing prostate biopsies. A four gene panel was selected in the clinical study: HOXC4, HOXC6, TDRD1 and DLX1, referred to as QUATTRO. QUATTRO had a predictive accuracy for the diagnosis of Gleason score ≥ 7 of 0.783; which was evidently higher than for sPSA (0.720) and ProgenSA PCA3 (0.688). When sPSA was added to QUATTRO, the predictive accuracy increased to 0.818 (compared to 0.789 when PCA3 was added to sPSA). In conclusion, we describe a stepwise development of a four gene-based test (QUATTRO), that was superior to sPSA and PCA3 to identify patients with Gleason score ≥ 7 prostate cancer.

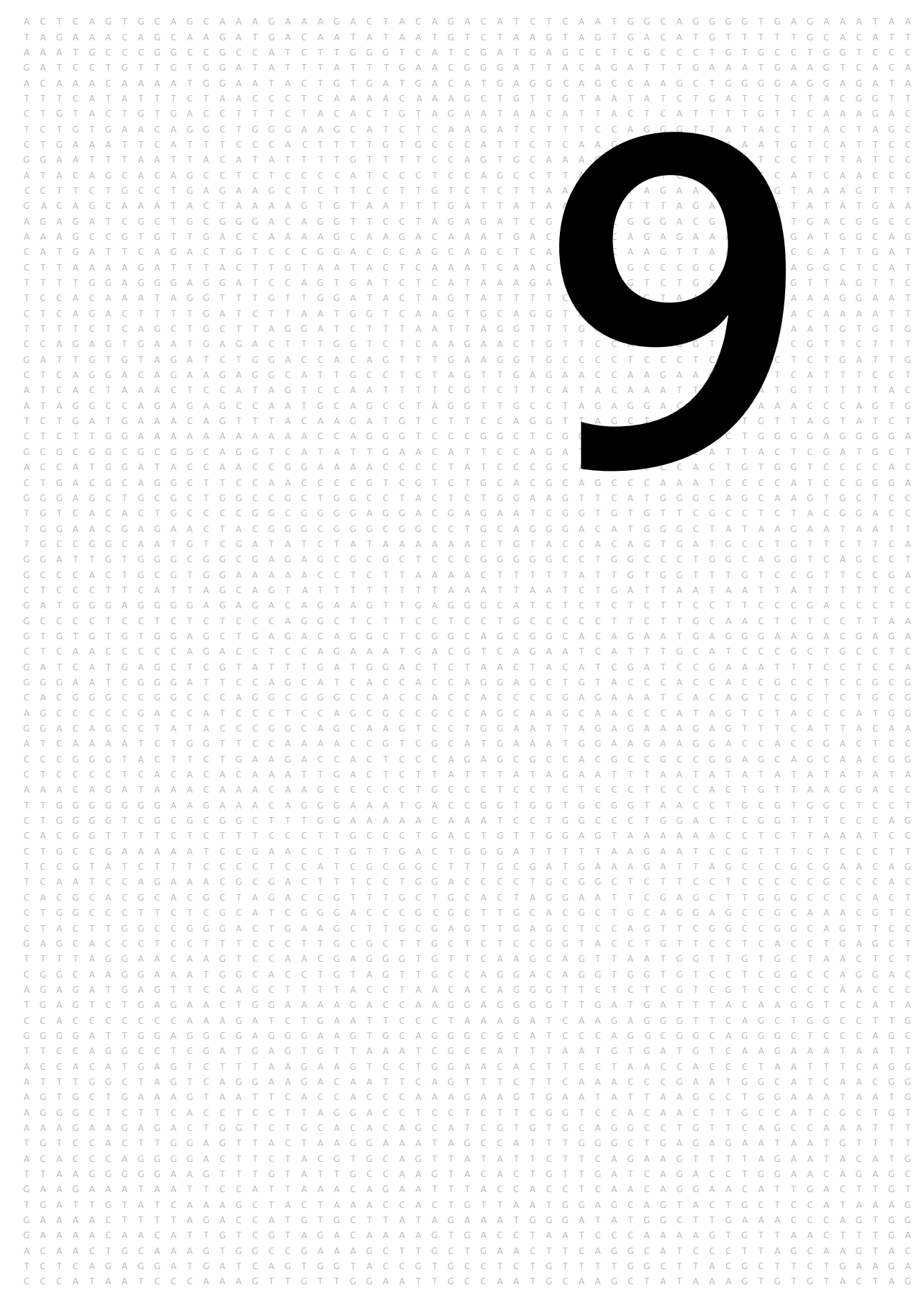
Patients with advanced prostate cancer who undergo chemical or surgical castration will ultimately experience a relapse, this state is called castrate-resistant prostate cancer (CRPC). Treatment options for CRPC patients are limited. The main issue in the management of CRPC and development of new therapies is the lack of surrogate endpoints for treatment response and survival. CTCs in the peripheral blood has been evaluated as a potential prognostic marker and predictor of overall survival. Little is known about prostate cancer specific biomarkers in CRPC patients as a surrogate test for CTCs. In **chapter 6**, we demonstrate the results of an exploratory study for the feasibility of a highly sensitive modified nucleic acid amplification assay to assess KLK3, PCA3 and TMPRSS2-ERG mRNA in the peripheral blood mononuclear cell fraction from CRPC patients. Blood samples from 20 CRPC patients were analyzed at four different time points (prior to docetaxel treatment, at 9 weeks, 27 weeks and 2 months after treatment). Blood was drawn once from three control groups (10 age-matched men, 10 men under 35 years of age and 12 women). All samples were analyzed for KLK3, PCA3 and TMPRSS2-ERG mRNA by using a quantitative nucleic acid amplification assay with gene-specific primers in the complementary DNA synthesis. At baseline, mRNA for KLK3 was detected in 17 (89%, 95% CI 76-100%), PCA3 in 10 (53%, 95% CI 30-75%) and TMPRSS2-ERG in 7 of 19 evaluable patients (37%, 95% CI 15-59%). In contrast, the blood samples from all 32 healthy volunteers were reproducible negative for all markers. In response to docetaxel treatment, KLK3 levels decreased in 80% (95% CI 60-100%), PCA3 in 89% (95% CI 68-100%) and TMPRSS2-ERG in 86% (95% CI 60-100%) of patients. In conclusion, the feasibility of a highly sensitive modified nucleic acid amplification assay to assess KLK3, PCA3 and TMPRSS2-ERG mRNA in the PBMC fraction from CRPC patients was demonstrated. Moreover, response of these markers to systemic treatment was shown. Molecular analysis of prostate (cancer) specific transcripts may be a surrogate for CTCs and monitoring systemic disease activity.

This thesis shows different applications of prostate cancer biomarkers: selecting patients that require MRI, selecting patients for (repeat) prostate biopsies and monitoring systemic disease activity in CRPC patients. Most importantly, we present the discovery and development of a four gene based urine test, QUATTRO, that was superior to serum PSA and PCA3 to identify patients with Gleason score ≥ 7 prostate cancer. This could be an important step forward in the ongoing worldwide search for reliable prognostic prostate cancer biomarkers.

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Prostaatkanker is de op een na meest gediagnosticeerde vorm van kanker bij mannen. De gouden standaard om de diagnose prostaatkanker te stellen is de histopathologische beoordeling van prostaatbiopten, een invasieve procedure met aanzienlijke morbiditeit. Gelokaliseerd prostaatkanker is meestal asymptomatisch, waardoor de indicatie voor prostaatbiopten gesteld wordt op basis van het serum PSA (prostaat specifiek antigeen) en rectaal toucher. **Hoofdstuk 1** is de algemene introductie en een overzicht van de inhoud van dit proefschrift.

Hoofdstuk 2 geeft een kort overzicht van de historie van biomarkers voor prostaatkanker, een overzicht van de huidige biomarkers voor prostaatkanker PSA en PCA3; en een overzicht van de potentiële nieuwe biomarkers voor prostaatkanker (weefsel markers, bloed markers en urine markers): TMRSS2-ERG, KI-67/MIB1-Labeling index, PTEN, E-cadherin, EZH2, neuroendocrine fenotype, microRNAs en circulerende tumor cellen (CTCs).

In de wereldwijde zoektocht naar betrouwbare biomarkers zijn urinemarkers veelbelovend gebleken. Prostaatkankercellen kunnen invaderen en migreren in de klierbuizen, die uitmonden in de plasbuis. Om de prostaatcellen te mobiliseren naar de plasbuis, dient een standaard rectaal toucher uitgevoerd te worden. De eerste portie urine die na het rectaal toucher wordt uitgeplast, bevat de hoogste concentratie prostaat- en urethracellen. Het urinemonster bevat zowel prostaattumorcellen als niet-neoplastische prostaatcellen. De ProgenSA PCA3 test is goedgekeurd door de FDA en de eerste urinetest voor prostaatkanker beschikbaar voor klinisch gebruik. PCA3 is in normaal en BPH (benigne prostaathypertrofie) prostaatweefsel aanwezig in zeer lage hoeveelheden. In prostaatkankerweefsel is er een overexpressie van PCA3 mRNA. De huidige indicatie voor de PCA3-test is het bepalen van het beleid om herhaalbiopten te nemen. Daarnaast wordt in steeds meer recentere studies aangetoond dat PCA3 ook ingezet kan worden om te bepalen een eerste biopst te nemen. Daarnaast wordt de multiparametrische MRI steeds vaker gebruikt in het diagnostische proces van prostaatkanker. De MRI kan met hoge mate van betrouwbaarheid prostaatkanker detecteren. Echter, door hogere kosten (vergeleken met echogeleide biopten) en beperkte capaciteit is het niet mogelijk om elke man met een indicatie voor prostaatbiopten MR-geleide biopten aan te bieden. Een test welke patiënten kan selecteren met een indicatie voor een MRI van de prostaat, en indien nodig daaropvolgend MR-geleide biopten, zou dus zeer wenselijk zijn. In **hoofdstuk 3** evalueren we de klinische waarde van de PCA3 test: de voorspellende waarde voor biopsie uitslag, Gleason score en MRI uitslag. Dit werd onderzocht in een retrospectieve studie van 591 mannen die allen een ProgenSA PCA3 test hadden gehad

in het Universitair Medisch Centrum Nijmegen St. Radboud. Er waren prostaatbiopsien verricht bij 290 mannen en een multiparametrische MRI bij 163/591 mannen. De PCA3 score had een hoog voorspellende waarde voor biopsie uitslag ($p < 0.001$), er was geen correlatie met Gleason score ($p = 0.194$). De PCA3 score van mannen met een verdacht gebied voor prostaatkanker op de MRI was significant hoger ($p < 0.001$) dan bij mannen zonder een verdacht gebied voor prostaatkanker op de MRI (52 vs. 21). Dit suggereert dat de PCA3 test patiënten zou kunnen selecteren met een indicatie voor een MRI van de prostaat.

TMPRSS2-ERG is een potentiële urine biomarker voor prostaatkanker, zeer specifiek voor prostaatkanker. Bij ongeveer de helft van de prostaatkanker patiënten is TMPRSS2 gefuseerd met ERG (genfusie). De urinetest heeft een sensitiviteit van 37% en een specificiteit van 93% voor het voorspellen van prostaatkanker in prostaatbiopsien. Gezien het heterogene karakter van prostaatkanker zal in de toekomst een biomarker panel de voorkeur hebben boven het gebruik van maar 1 biomarker. **Hoofdstuk 4** presenteert de resultaten van een grote prospectieve multicenter studie waarin de diagnostische en prognostische waarde van PCA3 en TMPRSS2-ERG werden geëvalueerd, zowel als individuele markers als een gecombineerd panel. Zowel PCA3 als TMPRSS2-ERG hadden een significant voorspellende waarde ($p < 0.001$ en resp. $p = 0.002$) in aanvulling op de ERSPC prostaatwijzer parameters (serum PSA, rectaal toucher, TRUS, prostaat volume). De voorspellende waarde voor prostaatkanker nam toe van 0.799 (ERSPC prostaatwijzer), naar 0.833 (ERSPC prostaatwijzer + PCA3) naar 0.842 (ERSPC prostaatwijzer + PCA3 + TMPRSS2-ERG). De sensitiviteit van PCA3 nam toe van 68% naar 76% wanneer werd gecombineerd met TMPRSS2-ERG. TMPRSS2-ERG had significant voorspellende waarde in aanvulling op de ERSPC prostaatwijzer parameters om biopsie Gleason score ($p < 0.001$) en klinisch tumor stadium te voorspellen ($p = 0.023$), PCA3 niet. Concluderend laat deze studie zien dat TMPRSS2-ERG gecombineerd met PCA3 significant voorspellende waarde had in aanvulling op de ERSPC prostaatwijzer parameters. TMPRSS2-ERG had prognostische waarde, en PCA3 niet.

De ProgenSA PCA3 test is waardevol in de diagnostiek naar prostaatkanker, echter de prognostische waarde lijkt beperkt. Biomarkers die patiënten met klinisch significant prostaatkanker kunnen identificeren zijn dus nog steeds hard nodig. In **hoofdstuk 5** beschrijven we de veelbelovende ontdekking en ontwikkeling van een 4 genen biomarker panel urine test met significant prognostische waarde. Middels microarray analyses op prostaatweefsel werden 46 potentiële markers geselecteerd. Vervolgens werden met Taqman low density arrays (TLDA) de 10 meest veelbelovende markers geïdentificeerd

(HOXC4, HOXC6, DLX1, TDRD1, ONECUT2, NKAIN1, MS4A8B, PTPRT, RRM2 en ACSM1). Hierbij werd de hypothese geformuleerd dat een combinatie van deze 10 biomarkers superieur is aan PSA en PCA3 om patiënten met Gleason score ≥ 7 prostaatkanker te identificeren. Deze hypothese werd getest in een prospectieve multicenter studie. Urinemonsters werden verzameld en getest van 358 mannen die prostaatbiopsie ondergingen. In deze klinische studie werd een 4 genen panel geselecteerd: HOXC4, HOXC6, DLX1 en TDRD1; genaamd QUATTRO. QUATTRO had een voorspellende waarde voor de diagnose Gleason score ≥ 7 prostaatkanker van 0.783; duidelijk hoger dan voor serum PSA (0.720) en ProgenSA PCA3 (0.688). Wanneer QUATTRO werd gecombineerd met serum PSA, nam de voorspellende waarde toe tot 0.818 (vergeleken met 0.789 voor de combinatie serum PSA + PCA3).

Patiënten met vergevorderd prostaatkanker en medicamenteuze of chirurgische castratie, zullen uiteindelijk progressie van de ziekte krijgen, dan spreken we van castratie resistent prostaatkanker (CRPC). De behandelmogelijkheden voor CRPC patiënten zijn zeer beperkt. Het grootste gebrek bij de behandeling van CRPC patiënten en de ontwikkeling van nieuwe therapieën is het gebrek aan een betrouwbaar surrogaat eindpunt voor respons op behandeling en overleving. CTCs gemeten in het bloed blijken uit eerdere studies een potentiële prognostische marker en voorspeller van overleving. Er is nog zeer weinig bekend over prostaat(kanker) specifieke biomarkers in CRPC patiënten als mogelijke surrogaat marker voor CTCs. In **hoofdstuk 6** beschrijven we de resultaten van een exploratieve studie naar de mogelijkheid van een nieuw ontwikkelde assay om KLK3 (PSA), PCA3 en TMPRSS2-ERG mRNA te meten in het bloed van CRPC patiënten. Bloedmonsters werden afgenomen van 20 CRPC patiënten op 4 verschillende momenten gedurende de behandeling met Docetaxel chemotherapie (dag 0, week 9, week 27 en 2mnd na behandeling). Er werd ook bloed afgenomen van 3 gezonde controle groepen (10 leeftijd-gematchte mannen, 10 mannen <35jaar, 12 vrouwen). Alle bloedmonsters werden geanalyseerd op KLK3, PCA3 en TMPRSS2-ERG mRNA met een kwantitatieve nucleïnezuur amplificatie assay met gen-specifieke primers. Bij start van de behandeling werd mRNA voor KLK3 gedetecteerd in 17 (89%), PCA3 in 10 (53%) en TMPRSS2-ERG in 7/19 evalueerbare patiënten (37%). Alle samples van de controle groepen waren negatief voor alle markers. Tijdens de Docetaxel behandeling daalden KLK3 spiegels in 80%, PCA3 spiegels in 89% en TMPRSS2-ERG spiegels in 86% van de patiënten. Concluderend laten we in deze studie zien dat het met deze assay met gen-specifieke primers mogelijk is om KLK3, PCA3 en TMPRSS2-ERG mRNA in perifere bloedmonsters van CRPC patiënten te meten. Ook laten we de reactie van deze markers op Docetaxel behandeling zien. De moleculaire analyse van prostaat(kanker) specifieke markers zouden mogelijk systemische ziekte activiteit

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kunnen monitoren en als surrogaat markers voor CTCs kunnen dienen, echter dit behoeft verder onderzoek.

Dit proefschrift laat verschillende toepassingen van biomarkers voor prostaatkanker zien: het selecteren van patiënten voor een MRI van de prostaat, selecteren van patiënten voor (herhaal) bipten van de prostaat en het monitoren van systemische ziekte activiteit bij CRPC patiënten. Meest belangrijk is de veelbelovende ontdekking en ontwikkeling van een nieuwe urine test: het 4 genen panel QUATTRO, met voorspellende waarde voor de diagnose Gleason score ≥ 7 prostaatkanker, superieur aan serum PSA en PCA3. Dit is een belangrijke stap voorwaarts in de wereldwijde zoektocht naar betrouwbare en prognostische biomarkers voor prostaatkanker.

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Curriculum vitae

DANKWOORD

In 2009 begon mijn project, en nu is het af! Ik ben erg blij dat ik deze kans gekregen heb, en ik had het zeker niet willen missen. Ik ben ervan overtuigd dat ik hierdoor een betere arts ben geworden. Het is namelijk essentieel om kennis van de wetenschap te hebben, alleen dan kun je nieuwe ontwikkelingen in de medische wereld op waarde schatten en op juiste wijze implementeren. Mijn dank gaat uit naar velen die hebben bijgedragen aan de totstandkoming van dit proefschrift, in het bijzonder de volgende mensen.

Prof. dr. J.A. Schalken, beste Jack. Aan jou heb ik zoveel te danken. We ontmoetten elkaar voor het eerst op de EAU in Stockholm in 2009. Ik kende je nog niet, maar ik had je artikelen over PCA3 gelezen en wist dat ik een promotietraject bij jou wilde doen. Dat heeft me geen moment teleurgesteld. Met veel toewijding en enorm veel kennis van zaken heb je me begeleid. Het was altijd weer intrigerend als ik met nieuwe ideeën bij jou kwam, en dan bleek dat jij allang tien stappen vooruit was. Daarnaast was het altijd zo onwijs gezellig tijdens de koffiepauzes, ook al ging het vaak over sport. Als pater familias aan het hoofd van de tafel en oprecht geïnteresseerd in iedereen aanwezig. Tijdens congressen in het buitenland hoefde ik niet veel uit te leggen, als ik vertel dat ik voor 'Jack Schalken' werk, dan weet men genoeg. Heel knap wat je allemaal bereikt hebt, en de persoonlijke manier waarop je leiding geeft aan het lab. Ik ben trots dat ik voor je heb mogen werken. Hopelijk kan ik vanuit de kliniek nog projecten met je blijven doen. En Anja, jij natuurlijk ook bedankt voor je betrokkenheid en al je gezelligheid!

Prof. dr. P.F.A. Mulders, beste Peter. Ook aan jou heb ik veel te danken. Dank voor de mogelijkheid om dit project te kunnen doen. En in de 2 jaar dat ik bij jullie heb gewerkt, vond ik het erg leuk dat je me zo hebt betrokken bij de kliniek. Elke week prostaatbiopsien verrichten, en meewerken op de 'prostaatkanker poli'. Dat was een goede combinatie voor mij. Verder de altijd kritische blik en de motiverende woorden bij de besprekingen hebben ervoor gezorgd alles naar een hoger plan getild werd. Dank voor alles!

Mijn copromotor, Dr. I.M. van Oort. Beste Inge, dank voor je begeleiding! Serieuze zaken bespreken tijdens de besprekingen, mij onder je hoede nemen samen met Michiel Sedelaar op de prostaatkanker poli en de gezelligheid daaromheen, het was een mooie tijd!

De leden van de manuscriptcommissie, Prof. dr. A.J. van Gool, Prof. dr. I.J. De Jong en Prof. dr. C.G.J. Sweep wil ik bedanken voor hun tijd en bereidheid mijn proefschrift te beoordelen.

Dit proefschrift was er waarschijnlijk nooit geweest zonder de hulp van Pieter Minnee en Toine van der Heijden. Jullie beiden hebben mij in contact gebracht met Jack, een essentiële eerste stap. Dank voor jullie vertrouwen in mij!

Alle collega arts onderzoekers urologie die de afgelopen jaren in het Radboud UMC hebben gewerkt: Harm, Martine, Jos, Minja, Ruben, Dick, Frank, Rianne, Luc, Siebren, Max, Stijn, Tom, Boy en Esther. Wat was het altijd gezellig, en wat hebben we een mooie tijd gehad. Borrelen in de Aesculaaf, bowlen in Big Lebowski stijl (veel White Russians) en alle buitenlandse congressen!

Siebren, erg leuk dat je mijn opvolger bent geworden! Dank voor de fijne samenwerking, en ik kijk al uit naar de dag dat jij gaat promoveren.

In het bijzonder wil ik Jos, Harm en Martine bedanken. Dankzij jullie voelde ik me meteen thuis in Nijmegen. Vele avondjes tripel bier drinken, en mountainbiken met z'n allen. Het laatste weekendje mountainbiken in de Ardennen was weer een groot succes (op bandenplakken na..), op naar ons lustrum volgend jaar!

Heel veel dank aan alle collega's op het Laboratorium Experimentele Urologie van het Radboud UMC. De ontspannen manier waarop er wordt samengewerkt, eindeloos beschikbare kennis en behulpzaamheid. Daarnaast alle gezelligheid tijdens de koffiepauzes en in de zomer cocktails drinken op het dak van NCMLS gebouw. Ik had me geen betere tijd kunnen wensen. Met een beetje weemoed denk ik aan jullie terug. Kees, dank voor je legendarische foute kroegentocht door Nijmegen! Guru Gerald, dank voor de spannende wedstrijdes wordfeut. Onno, Dorien en Nicoline dank voor de fantastische sushi avondjes met ons favoriete programma op tv!

Ook heel veel dank voor alle mensen van NovioGendix: Daphne, Frank, Sander, Hans, Kim, Pim, Rob en Willem. Ik heb het altijd een erg prettige samenwerking gevonden, en heb veel bewondering voor jullie ervaring en het hoge niveau waarop jullie werken. Indrukwekkend wat jullie allemaal kunnen. En wat hebben jullie een heerlijke vlam tosti's in de kantine, die hebben we in Amsterdam niet!

Veel dank aan het team urologie van het UMC Radboud. De getoonde interesse in mijn onderzoek en alle hulp bij het includeren van patiënten in mijn studies.

Heel veel dank voor de bijdrage van alle coauteurs: dr. Erik Cornel, Prof. dr. de Reijke, dr. Henk Vergunst, dr. Paul Kil, Ben Knipscheer, dr. Christina Hulsbergen - van de Kaa, Lieske Wierenga, dr. Jurgen Futterer en Prof. dr. J. Barentsz. Dank voor het includeren van de vele patiënten en voor jullie adviezen. Daarnaast veel dank voor alle assistentes van de urologie poli's waar ik langs kwam om patiënten gegevens te verzamelen. Dank voor jullie hulp bij het opvragen van alle papieren statussen, de gezelligheid en voor de koffie!

In het bijzonder wil ik Prof. dr. Th. M. de Reijke bedanken. Dank dat u vertrouwen in mij heeft, en mij heeft aangenomen voor de opleiding tot uroloog.

Pieter Minnee en Goos Takens heel veel dank voor de fantastische ANIOS tijd die ik bij jullie heb gehad! Jullie hebben mij definitief de keuze doen maken voor dit mooie vak. Alle facetten van het vak kwamen aan bod, en ik heb veel ervaring op kunnen doen. Ook dank aan alle urologie assistentes van t Lange Land voor alle gezelligheid. Annemiek, ik denk nog regelmatig terug aan onze "vrijdag, vas-dag", de hele dag sterilisaties uitvoeren, heerlijk!

Alle chirurgen en (oud) collega arts assistenten uit het Westfriesgasthuis te Hoorn, dank voor de twee ontzettend leerzame vooropleidingsjaren en alle gezelligheid!

Alle urologen en collega arts assistenten in het OLVG te Amsterdam. Bedankt voor alles dusver, op naar een mooi tweede jaar!

Lieve Stephanie en Jos, ik ben héél blij dat jullie vandaag mijn paranimfen zijn. Met jullie aan mijn zijde komt het goed. Stephanie, we kennen elkaar al zo lang, en ik vind het super dat we beiden voor dit mooie vak hebben gekozen! Op nog vele mooie momenten samen. Jos, we hebben twee jaar lang elke dag naast elkaar gezeten en dan leer je elkaar goed kennen. Wat was het altijd gezellig, goede muziekjes, 'weetjes van de dag', discussies tijdens de koffiepauzes, de vele borrels, snorrels, en de altijd weer legendarische congressen. Een top tijd was het!

Zonder steun van mijn lieve vrienden en familie was het mij zeker niet gelukt dit proefschrift te schrijven. Lieve Oysters, dank voor jullie begrip en interesse in mijn onderzoek. En met name de nodige ontspanning, nu heb ik nog meer tijd om nog meer mooie dingen met jullie te beleven! Clubbies, helaas geen wekelijkse clubavonden meer, maar wel altijd weer fijn jullie te zien. Dank voor alle gezelligheid!

Lieve mama, Claire & Pieter en Mathé & Ellis, lieve familie. Vanzelfsprekend zijn jullie heel belangrijk voor mij. Dank voor al jullie onvoorwaardelijke steun en interesse in alles wat ik doe.

Lieve papa, in gedachten ben je altijd bij me.

Lieve Volkert, wat ben ik blij dat we elkaar gevonden hebben! Dank voor al je liefde, geduld, steun en afleiding bij het afronden van m'n proefschrift. Het leven met jou is heel mooi!

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¹These authors equally contributed to this work.

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QUATTRO, a four gene prognostic biomarker panel for prostate cancer.

Submitted.

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CURRICULUM VITAE



Gisèle Leyten studied medicine at the University of Groningen. Gisèle first developed her interest in urology during her internships at the Deventer Hospital. After having obtained the medical degree in 2007, she worked at the Berekum Holy Family Hospital in Ghana for 4 months as a medical doctor under supervision of a Dutch medical doctor specialized in tropical medicine. When back in the Netherlands, she worked as a resident not in training (ANIOS) at the department of General Surgery at the Meander Medical Centre in Amersfoort, and at the department of Urology of 't Lange Land Ziekenhuis in Zoetermeer.

After this period of clinical experience, she worked 2 years as a PhD candidate at the department of Experimental Urology at the Radboud University Medical Centre in Nijmegen. She worked under the supervision of Prof. dr. J.A. Schalken and Prof. dr. P.F.A. Mulders on a research project which focusses on the use of biomarkers for prostate cancer and the search for prognostic biomarkers for prostate cancer. During this period, she attended several conferences and courses in the Netherlands and abroad. In 2012, she started the Urology Residency Training Program. From 2012-2014, she worked at the department of General Surgery at the Westfriesgasthuis in Hoorn. Currently, she works at the department of Urology at the Onze Lieve Vrouwe Gasthuis in Amsterdam. In 2016, she will start working at the department of Urology at the Amsterdam Medical Centre, for the last two years of the training program.

Gisèle H.J.M. Leyten. Born on 19 September 1983 (Swifterbant, the Netherlands)

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